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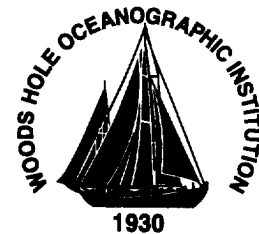
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DOCTORAL DISSERTATION

Characterization of P-glycoprotein Expression as a Multixenobiotic Resistance Mechanism in Fish

by

Shannon Mala Bard

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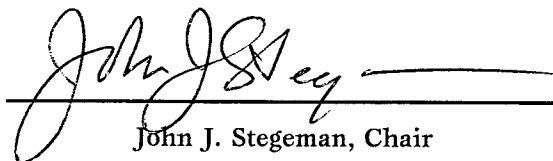Woods Hole, Massachusetts 02543

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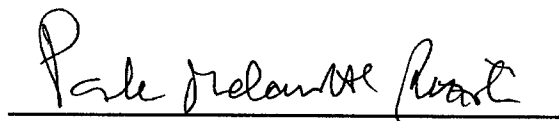
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Characterization of P-glycoprotein expression as a
multixenobiotic resistance mechanism in fish

by

Shannon Mala Bard

B.S., Biological Sciences, Stanford University, 1994

Submitted in partial fulfillment of the degree of

Doctor of Philosophy

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

and the
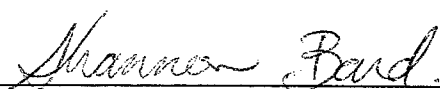
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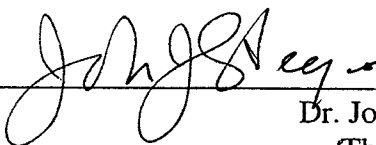
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Characterization of P-glycoprotein expression as a multixenobiotic resistance mechanism in fish

by

Shannon Mala Bard

Submitted to the Department of Biology and the Joint Program in Oceanography

on October 6, 2000 in Partial Fulfillment of the Requirements for the Degree of Doctor of

Philosophy in Biological Oceanography

ABSTRACT

   Transmembrane P-glycoproteins (P-gps) are responsible for multidrug resistance (MDR) phenotypes in tumor cell lines. P-glycoproteins function as energy dependent efflux flippases that prevent the cellular accumulation of a wide variety of compounds. We characterized P-gp expression in populations of several fish species exposed in their natural habitat to environmental contaminants which may be P-gp substrates/inducers. We evaluated whether P-gp activity may be implicated in this multixenobiotic resistant phenotype.

   In winter flounder (*Pleuronectes americanus*) with contaminant–associated liver tumors, P-gp was highly expressed in bile canaliculi of non-tumorous liver surrounding cholangiocellular carcinoma, but was not detected within tumors. Cellular stress caused by impaired bile elimination may be responsible for elevated P-gp.

   Killifish (*Fundulus heteroclitus*) from a contaminated field sites had higher intestinal P-gp and lower hepatic P-gp than control killifish. In contaminated fish, elevated intestinal P-gp may provide a barrier against absorption of P-gp substrates/inducers thus limiting the amount of these compounds exported to the liver.

   We investigated whether P-gp might be involved in induction of cytochrome P4501A (CYP1A). Although CYP1A and P-gp were both elevated in oil exposed blennies (*Anoplarchus purpurescens*), there was no induction of P-gp in blennies exposed to ß-naphthoflavone nor in killifish exposed to 2,3,7,8-tetrachlorodibenzofuran, both CYP1A inducer. Thus in fish, P-gp expression is not regulated by the aryl hydrocarbon receptor pathway.

   We developed a protocol for an *in vivo* assay to simultaneously evaluate P-gp-mediated transport of a model substrate, rhodamine B (rhB), in multiple organs of

killifish. Our results indicate that P-gps play a major role in transport of xenobiotics in fish especially in liver, brain, and ovary. Using this assay, we assessed whether the common environmental contaminant and carcinogen benzo[a]pyrene (B[a]P) is a P-gp substrate. We show that B[a]P and/or its CYP1A metabolites are not transported by P-gp in liver, brain, or ovary.

Thesis Supervisor: Dr. John J. Stegeman
Title: Senior Scientist

Acknowledgments

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Chapter 1: Introduction

P-glycoprotein expression as a multixenobiotic resistance mechanism in fish

Many aquatic species are able to survive in environments which contain high levels of multiple anthropogenic pollutants or natural product toxins (Bard, 1998; Cembella *et al.*, 1994.) This multixenobiotic resistance (MXR) phenomenon is similar to multidrug resistance (MDR) first observed in tumor cell lines resistant to anti-cancer drugs. Overexpression of a 170 kDa transmembrane P-glycoprotein (P-gp) was found to prevent the accumulation of cytotoxic drugs in resistant cells. MDR is of clinical importance because many human tumors have inherent or acquired P-gp-mediated drug resistance and do not respond to chemotherapy. P-gp is found endogenously in specialized epithelial tissues involved in secretion and excretion such as the mammalian gut, liver, and, kidney, as well as on endothelial cells of capillary blood vessels at the blood-brain barrier.

P-gp acts as an energy-dependent pump to translocate a wide variety of structurally and functionally diverse substrates. These compounds tend to be moderately hydrophobic, planar, natural products which are often substrates for or metabolites of detoxification enzymes such as cytochromes P450 (CYPs.) P-gps prevent the cellular accumulation of endogenous metabolites, phospholipids, and xenobiotics in exposed animals and cell cultures.

P-gp-like proteins have been described in a variety of aquatic organisms including sponges, mussels, oysters, clams, worms, and fish. Both natural products and anthropogenic contaminants found in the marine environment appear to be substrates and

inducers of the multixenobiotic resistance transporter in aquatic organisms. These observations suggest that in addition to normal cell function, P-gp activity may contribute to the relative hardiness of some marine species exposed to xenobiotics. The induction of a multixenobiotic defense mechanism in organisms living in polluted environments may explain why contaminant spills cause more severe adverse effects at pristine sites than in already polluted areas.

Although P-gps have been intensively studied in relation to cancer biology using highly selected drug resistant cell lines and chemotherapeutic agents, less is known regarding multixenobiotic resistance in wild populations of organisms and environmentally relevant P-gp substrates. The purpose of this introduction is to place ecotoxicological data in context of the larger multidrug resistance field of study and then to outline the work that comprises this thesis.

Background: Multidrug resistance

Multidrug resistance is a phenomenon which was first observed in tumor cell lines selected for resistance to a single agent which developed simultaneous resistance to a wide variety of structurally and functionally unrelated antineoplastic drugs (Kessel *et al.*, 1968; Biedler *et al.*, 1975; Nielsen and Skovsgaard, 1992.) Human cancer patients were observed through the course of chemotherapy to become resistant to not only the initial drug but also a host of other antiproliferative agents to which they had not been previously exposed. Juliano and Ling discovered that a 170 kD P-glycoprotein (P-gp) was responsible for the resistance of some tumor cells to anti-cancer drugs (Juliano and Ling, 1976.) This phosphorylated and glycosylated P-glycoprotein is localized in the membrane of resistant cells. P-gp consists of a 1280 amino acid transmembrane dimer which acts as an energy dependent efflux pump to prevent the accumulation of drugs within the cell. Each half of the protein consists of 6 transmembrane regions, a

cytoplasmic domain and an ATP binding site (Chen *et al.*, 1986.) Recent reviews have detailed drug resistance (Skovsgaard et al 1994; Ambudkar *et al.*, 1999); P-gp structure and evolutionary homologies (Bosch and Croop, 1998); pharmacology of MDR chemosensitizers (Ford and Hait, 1990); assays to quantify P-gp (van der Heyden *et al.*, 1995); P-gp function (Borst and Schinkel, 1997.)

P-glycoproteins undergo considerable post-translational modification including glycosylation and phosphorylation. Three carbohydrate side chains are located in the first extracellular loop of MDR1. The absence of N-glycosylation sites reduces drug transport efficiency although substrate specificity is not altered (Schinkel *et al.*, 1993.). This observation suggests that N-glycosylation contributes to proper routing of the protein to the plasma membrane or stability of P-glycoprotein, but not directly to drug transport. P-glycoproteins are phosphorylated at many threonines and serines (Gottesman and Pastan, 1993) but how phosphorylation relates to function is currently unclear. P-gp phosphorylation by protein kinase A and C have been observed to slightly enhance ATPase and substrate transport activities (Shapiro and Ling, 1995.) However drug transport activity was not altered in P-gps whose Ser/Thr were replaced by the unphosphorylatable Ala or by Asp (whose negative charge mimics that of phosphorylated Ser/Thr) (Germann *et al.*, 1996; Goodfellow *et al.*, 1996.)

Highly conserved MDR genes have been described in diverse taxa including rodents (Ng *et al.*, 1989), chicken (Edelmann, 1999) fruit fly (Dressen *et al.*, 1988), nematode worm (Broeks *et al.*, 1995), plants (Dudler and Hertig, 1992; Wang *et al.*, 1996; Davies *et al.*, 1999D), archaebacteria and bacteria (Felmlee *et al.*, 1985), and several marine organisms including winter flounder *Pleuronectes americanus* (Chan *et al.*, 1992), mummichog or killifish *Fundulus heteroclitus* (Cooper *et al.*, 1998) and a marine worm *Urechis caupo* (Toomey, 1995.) Two classes of mammalian MDR genes

which code for functionally different P-glycoproteins have been well characterized. Refer to Figure 1 for multiplicity and nomenclature of P-gp genes across taxa. Class I genes (human MDR1 and mdr1a and mdr1b in rats) confer constitutive and inducible drug and xenobiotic resistance while class II genes encode a constitutive, apparently uninducible phosphatidylcholine translocator (van Helvoort *et al.*, 1996.) A recently described, related 160 kD P-glycoprotein, dubbed Sister of P-gp or spgp, is localized to the bile canalicular microvilli and subcanalicular vesicles of hepatocytes (Childs *et al.*, 1995, Gerloff *et al.*, 1998) with lower expression detected in the brain grey cortex, small- and large-gut mucosa (Török *et al.*, 1999.) Spgp is believed to be the major canalicular bile salt export pump of the mammalian liver which transports primary bile salts such as taurocholate (Gerloff *et al.*, 1998.) Spgp-related proteins have been indicated in humans, rats, mice, chicken, turtle, winter flounder and *Fundulus heteroclitus* (Childs *et al.*, 1995; Cooper *et al.*, 1998; Gerloff *et al.*, 1998.)

Two fish P-glycoprotein (fpgp A and B) genes have been partially cloned in winter flounder and mummichog (Chan *et al.*, 1992; Cooper *et al.*, 1998.) Fpgp A corresponds to spgp while fpgp B is related to both class I and class II mammalian P-glycoproteins (Cooper, 1996; Childs, 1998 personal communication.) The partially sequenced *U. caupo* P-glycoprotein has approximately 50% sequence homology to mammalian class I, class II, and spgp (Toomey, 1995.) The anti-P-glycoprotein C219 monoclonal antibody recognizes an internal, highly conserved amino acid sequence near the nucleotide binding domain common to all P-glycoprotein isoforms whose sequence is known (Kartner *et al.*, 1985; Endicott and Ling, 1989.) Studies of marine invertebrates have identified P-glycoproteins with mAb C219 and have demonstrated functional homology to the xenobiotic resistance transporter, but this information is insufficient to definitively classify these P-glycoproteins by mammalian nomenclature. Furthermore,

many of the mammalian studies cited in this review do not distinguish between P-glycoprotein isoforms.

P-gps are members of the ABC (ATP-binding cassette) superfamily of structurally similar transmembrane transport proteins present in eubacteria, eukaryotes, and recently described in Archaea (Jovell *et al.*, 1996; Koonin *et al.*, 1997; Xavier *et al.*, 1996 (recently reviewed by Ambudkar *et al.*, 1999.) In eukaryotes, ABC transporters function to remove molecules from the cytoplasm and export them either through the cell cytoplasmic membrane or through organelle membranes (Saurin *et al.*, 1999.) Prokaryotes have two types of ABCs involved in either export or uptake of molecules (Saurin *et al.*, 1999.) ABC transporters include hCFTR, the human cystic fibrosis transmembrane conductance regulator (Higgins *et al.*, 1986), *pfmdr* chloroquine resistance in *Plasmodium falciparum* the organism responsible for malaria (Foote *et al.*, 1989), *STE6* lipopeptide transporter in *Saccharomyces cerevisiae* (McGrath and Varshavsky, 1989), human LRP the lung resistance protein (Scheper *et al.*, 1993), and mammalian MRP the multidrug-resistance-associated protein (Cole *et al.*, 1992.)

LRP and MRP provide MDR in some tumor cell lines which do not overexpress P-gp. The 110 kd LRP is the major cytoplasmic vault protein involved in nuclear-cytoplasmic transport (Scheffer *et al.*, 1995.) MRPs are 190 kD glycoproteins with three multispanning transmembrane domains compared to two in MDR1 P-glycoprotein and these protein families share between 14-25% amino acid identity (Cole *et al.*, 1992, Keppler and König, 1997.) The human MRP family contains at least six members which have on average 50% amino acid identity between them (Keppler and König, 1997; König *et al.*, 1999): MRP1 gene encodes multidrug-resistance-associated protein in extrahepatic tissues (Cole *et al.*, 1992); MRP2 or cMOAT encodes the canalicular multispecific organic anion transporter found in liver and kidney (Buchler *et al.*, 1996;

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Schaub *et al.*, 1997); basolateral MRP3 (BLMRP) is expressed in the basolateral membrane of hepatocytes, colon, pancreas and kidney (König *et al.*, 1999); MPR6 is expressed in liver and kidney but function is unknown (Kool *et al.*, 1999); and two additional homologues, MRP4 and MRP5 whose distribution and function is unknown (Kool *et al.*, 1997.) MRP1 and MRP2 are responsible for export of amphiphilic anions and anionic conjugates formed via metabolism by phase II enzymes (Cole *et al.*, 1994, Zaman *et al.*, 1995), for example, cholestatic glucuronidated steroids (Loe *et al.*, 1996) and leukotriene C4 glutathione conjugate, an arachidonic acid metabolite important for regulation of blood pressure (Lautier *et al.*, 1996.) MRPs are believed to be the previously described GS-X pump a member of the phase III drug metabolizing system (Ishikawa, 1992 .) MRP-mediated transport may both complement and supplement P-gp efflux due to some substrate overlap between the two systems (Loe *et al.*, 1998; Robson *et al.*, 1998.)

Mechanism of transport

The mechanism by which P-glycoprotein transports xenobiotics is unknown. Initially, some groups presented evidence that P-gp functions as both an ATP channel (Abraham, 1993) and a Cl- channel (Valverde *et al.*, 1992.) These claims of channel activity have been refuted (Boyum and Guidotti, 1997), however, data suggests that P-gp may regulate the activity of other Cl- channels in some cell types and be involved in osmotic control (Higgins, 1995.) The classic pump model of membrane transporters is insufficient to explain all aspects of P-gp transport (Sharom, 1997.) In the classical model, the membrane transporter forms an aqueous pore which undergoes a conformational change in order to move a hydrophilic substrate from the aqueous phase cytosol to the aqueous phase extracellular space. Although this model may not be

rejected outright, several modifications are required since the substrates are generally hydrophobic and transporter-substrate binding occurs in the lipid phase of the membrane.

Higgins and Gottesman proposed the *hydrophobic vacuum cleaner* model in which drugs are transported from the plasma membrane to the extracellular medium (Higgins and Gottesman, 1992.) The model presents a two-tier recognition process: the primary determinant of substrate specificity is a compound's ability to intercalate into the membrane (hydrophobicity) and the following interaction of the compound with a relatively nonselective binding site on P-gp would be of secondary importance. But this model too was not entirely satisfactory in part because P-gp was shown to specifically bind to substrates with different specificities (Liu and Sharom, 1996.)

The currently favored model proposes that P-gp flips drugs from the inner to the outer leaflet of the plasma membrane against an intramembrane concentration gradient (Higgins and Gottesman, 1992.) The term *flippase* was originally coined to describe membrane enzymes that translocate phospholipids. Although the lateral mobility of phospholipids is high, movement between leaflets is low because the polar head group of the phospholipid has difficulty passing through the hydrophobic interior of the membrane. Flippases speed up this process. Support for this model was provided by Smit *et al.*'s discovery that mouse canalicular mdr2 facilitates the normal transport of phosphatidylcholine from the hepatocyte into the bile (Smit *et al.*, 1993.) Recent work with pig kidney cells transfected with class I and class II mdr cDNA constructs revealed that both isoforms can translocate short-chain fluorescent phospholipid analogs (van Helvoort *et al.*, 1996.) MDR1 is presumed to transport molecules which intercalate and introduce discontinuities into the bilayer, such as amphipathic drugs, by the same flippase mechanism (Higgins and Gottesman, 1992.)

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Recently, Shapiro and Ling presented evidence that P-glycoprotein contains at least two distinct substrate binding and transport sites and that these sites interact in a positively cooperative fashion (Shapiro and Ling, 1997.) They propose that one site dubbed R (for the substrate rhodamine 123 and anthracyclenes) binds one set of compounds and the other site H (for the substrate Hoechst 33342 and colchicine) binds a second set (Shapiro and Ling, 1998; Shapiro and Ling, 1997.) Each R substrate stimulates the P-glycoprotein-mediated transport of an H substrate and vice versa. Two R substrates would compete for the R site and inhibit binding and transport of the other. Competitive inhibition would also be observed between two H substrates or between substrates which can bind to both R or H sites. A third drug-binding site (for the substrates prazosin and progesterone) on the P-glycoprotein exerts a positive allosteric effect on drug transport by the H and R sites but is not thought to be capable of drug transport itself (Shapiro et al., 1999.) Shapiro and Ling's three-site model is consistent with previous observations of both competitive and non-competitive interactions between P-gp substrates.

P-glycoprotein substrates

The basis for the extremely broad substrate specificity of P-gp has not yet been elucidated. P-gp substrates differ in cytologic target, chemical structures, and properties (Endicott and Ling, 1989.) The only obvious physical similarities substrates share are being moderately hydrophobic, amphipathic (i.e. somewhat soluble in both lipid and water), low molecular weight, planar molecules with a basic nitrogen atom, cationic or neutral but never anionic, and natural products (Gottesman and Pastan, 1988; Endicott and Ling, 1989; Pearce et al., 1990; Gottesman et al., 1994) Refer to Figure 4 for the chemical structures of model MDR1 P-glycoprotein substrates. P-gp substrate chemotherapeutic drugs include colchicine, Vinca alkaloids (e.g. vinblastine, vincristine),

actinomycin D, taxol, epipodophyllotoxins (e.g. etoposide), and anthracyclines (e.g. doxorubicin.) Many natural product compounds are also transported by P-gp including calcium channel blockers (e.g. verapamil and dihydropyridines), antiarrhythmics (e.g. quinidine), antihyperteinsives (e.g. reserpine), steroids (e.g cortisol, dexamethasone, aldosterone), and antiparasitics (e.g. quinine, ivermectin) to list a small number. Several groups have reported P-gp structure-activity models to help predict which drugs will interact with P-gp (Zamora *et al.*, 1988; Bain *et al.*, 1997; Klopman *et al.*, 1997; Etievant *et al.*, 1998; Seelig, 1998.)

Evidence suggests that P-gp transports environmental contaminants (or possibly their phase I metabolites) 7, 12-dimethylbenz(a)anthracene (Phang *et al.*, 1993) and the pesticide endosulfan (Bain and LeBlanc, 1996.) Natural product screens have discovered numerous P-gp substrates derived from marine organisms including okadaic acid, the causative agent in human diarrhetic shellfish poisoning derived from the marine alga *Dinophysis* spp. (Suganuma *et al.*, 1988); calyculin A, a potent tumor promoter and protein phosphatase inhibitor isolated from the marine alga *Acetabularia* sp. (Chambers *et al.*, 1993); patellamide D a cyclic octapeptide isolated from the marine tunicate, *Lissaclinum patella* (Williams and Jacobs, 1993); dolastatins, small peptides isolated from the marine sea hare *Dolabella auricularia* (Aherne *et al.*, 1996); and lamellarins, polyaromatic alkaloids isolated from tunicates (genus *Didemnum*) (Quesada *et al.*, 1996.)

P-glycoprotein expression in normal tissues and tumors

P-glycoprotein expression is detected in three distinct subsets of normal human tissue involved in secretion, absorption or a barrier function, and in some tumors derived from these tissues: a subset of columnar epithelial cells, endothelial cells of capillary beds in specific anatomic locations, and placental trophoblasts. (The trophoblast is a thin layer of ectoderm that constitutes the wall of a mammalian blastula and is important for the

nutrition and implantation of the embryo.) P-gp overexpression in tumors prior to treatment is a poor prognosticator of chemotherapy success (van der Heyden *et al.*, 1995.)

Human MDR1 has been detected at high levels in liver, kidney, small bowel, colon, pancreas, adrenal cortex, placenta, testis-brain barrier, and blood-brain barrier (Fojo *et al.*, 1987; Sugawara *et al.*, 1988; Cordon-Cardo *et al.*, 1990; Sugawara, 1990.) MDR1 P-gp transports drugs and xenobiotics that are unmodified by phase II cellular metabolizing enzymes. Evidence suggests that MDR1 P-gp in the adrenal cortex is involved in transport of steroid hormones particularly glucocorticoids (Naito *et al.*, 1989; Ueda *et al.*, 1992; Wolf and Horwitz, 1992.) The MDR2 encoded P-gp is not involved in the transport of hydrophobic anticancer drugs. This isoform is highly expressed in the canalicular membranes of hepatocytes (Smit *et al.*, 1994) and has been detected at low levels in the spleen, B cells, heart and muscle (Buschman *et al.*, 1992.) Canalicular MDR2 is involved in the transport of phospholipids into bile (Smit *et al.*, 1993; van Helvoort *et al.*, 1996.) Sister of P-glycoprotein is localized in the canalicular microvilli and to subcanalicular vesicles of the hepatocytes and functions as the major canalicular bile salt export pump of mammalian liver (Childs *et al.*, 1995; Gerloff *et al.*, 1998.) Spgp expression has also been detected in the brain grey cortex, small and large gut mucosa by RT-PCR (Török *et al.*, 1999.)

P-glycoprotein function in fish

Although much work in the MDR field has been conducted in *in vitro* systems, the importance of P-gp activity in whole animals has not been entirely elucidated. Furthermore, few have investigated P-gp expression in natural populations (reviewed in Chapter 2). We characterized P-gp expression in populations of fish that are exposed in their natural habitat to environmental contaminants that may be P-gp substrates and/or

inducers. Our aim was to evaluate whether P-gp activity may be implicated in the multixenobiotic resistant phenotype exhibited by these fish. We examined several questions: first, whether P-gp expression in cholangiocellular carcinomas resembles classical mdr; second, whether P-gps in multiple organs of fish are induced by exposure to environmental xenobiotics; third, whether P-gps are involved in the induction of CYP1A; and finally we developed an assay to investigate P-gp mediated transport of xenobiotics *in vivo*.

First, we examined P-gp expression in liver of winter flounder that bore cholangiocellular carcinomas possibly caused by exposure to carcinogens at a contaminated Boston Harbor site (Chapter 3). In contrast to expectations based on the classical mdr phenotype of elevated P-gp levels being commonly observed within hepatocellular carcinomas in mammals and fish, P-gp was highly expressed in bile canaliculi of non-tumorous liver parenchyma surrounding the cholangiocellular carcinoma, but was only detected within the carcinoma of one individual. We suggest that cellular stress caused by impaired bile elimination, possibly linked to the presence of the cholangiocellular carcinomas in these fish, may be responsible for the elevated P-gp observed in the normal liver parenchyma surrounding tumors.

In a second study, we investigated whether levels of P-gp expression in the intertidal fish high cockscomb blenny (*Anoplarchus purpurescens*) might be altered by field and/or laboratory exposures to crude oil or pulp mill effluent (Chapter 4). Because candidate P-gp inducers present in these contaminant mixtures (such as polycyclic aromatic hydrocarbons (PAHs)) are known to induce the biotransformation enzyme

cytochrome P450 1A (CYP1A) (James and Bend, 1980; Lindstrom-Seppa, 1988), we also examined whether P-gp expression might be related to CYP1A induction. P-gp and CYP1A were both induced in blennies by some compounds in petroleum and unidentified xenobiotics at field sites. However, depurated blennies that were injected with the aryl hydrocarbon agonist ß-naphthoflavone showed an expected induction of CYP1A but no induction of P-gp. Furthermore, in our winter flounder study we found no correlation between hepatic CYP1A and P-gp expression in the examined populations (Chapter 3). And in a third study, killifish (*Fundulus heteroclitus*) injected with another model CYP1A inducer, 2,3,7,8-tetrachlorodibenzofuran (TCDF), showed no induction of P-gp (Chapter 5). These results suggest that in fish, P-gp expression is not regulated by the aryl hydrocarbon receptor pathway. Although CYP1A and P-gp are not coordinately regulated, these proteins may play complementary roles in cellular detoxification.

We investigated whether P-gps contribute to the xenobiotic resistant phenotype observed in a natural population of killifish exposed to planar halogenated aromatic hydrocarbons at the New Bedford Harbor Superfund site (NB) (Chapter 5). We examined P-gp expression in liver and intestine, the predominant organs involved in interactions with chemicals from the environment, in NB fish and fish from a reference site at Scorton Creek, Cape Cod (SC). Compared to freshly collect fish, P-gp expression in liver and intestine decreased in fish from both sites maintained in laboratory by day 8 but no further decrease was noted up to 78 days after collection. The lack of induction of P-gp in TCDF-treated fish and the fact that hepatic and intestinal P-gp levels did not further decrease after 8 days depuration suggest that the highly lipophilic contaminants

such as planar HAHs might not be responsible for P-gp induction. Freshly collected fish had different levels of P-gp expression in liver (SC>NB) and intestine (NB>SC). Elevated intestinal P-gp in NB fish might provide a barrier against absorption of P-gp substrates/inducers and thus limit the amount of these compounds exported to the liver, which might account for the lower hepatic P-gp levels in NB fish compared to SC fish. We suggest that these differences might also reflect differing environmental exposure to anthropogenic contaminants or microbial, algal, plant or other natural products via the sediment or diet at each site.

In addition to studying patterns of P-gp expression in different populations of fish, we were particularly interested in how P-gp affects the intracellular disposition and retention of xenobiotic *in vivo*. Whole animals studies are needed to elucidate how P-gp influences the toxicological effects of its substrates on, for example, tissue disposition, interactions with other xenobiotics, and expression of xenobiotic inducible metabolizing enzymes. Understanding the effect of P-gp activity on xenobiotic disposition at the organismal level is important for evaluating, for example, health risks of environmental contaminants to a human population. Model species must be selected for such studies when one is ethically prohibited from conducting this work in humans. The toxinicant exposure history of humans may more closely resemble that of coastal fish, which are continually challenged by exposure to water-borne anthropogenic contaminants and natural product toxins, than inbred rodents maintained in laboratories and fed clean food. Individual variability in P-gp activity, which has been observed in humans and fish (Chapter 5 & 6), may effect disposition and detoxification of xenobiotics and may be a

determinant of individuals' differences in pharmacological response to drugs and toxicological effects of environmental contaminants that are P-gp substrates. Such diversity in response cannot be examined in mdr knockout mouse models for obvious reasons.

We developed a protocol for an *in vivo* assay, and applied this assay to evaluation of P-gp-mediated transport of a model substrate, rhodamine B (rhB), in multiple organs of killifish (Chapter 6). Our results indicate that P-gps play a major role in transport of xenobiotics in fish, especially in liver, brain, and ovary. There are continuing questions concerning P-gp substrate specificity for environmental contaminants. Benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon (PAH), is a common environmental contaminant and known carcinogen (Buening *et al.*, 1978) that has been shown to induce P-gp in cell culture (Fardel *et al.*, 1996), and transport of B[a]P observed in several *in vitro* systems has been attributed to P-gp-mediated transport (Yeh *et al.*, 1992). Using a modified version of this assay, we evaluated whether *in vivo* inhibition of P-gp by a chemosensitizer drug (cyclosporin A (CsA)) altered the accumulation of radiolabeled ^{3}H-B[a]P in bile, liver, brain or ovary (Chapter 7). The highly lipophilic structure of B[a]P is not typical of P-gp substrates. However, the parent compound B[a]P may be oxidized through the action of the phase I enzymes, such as cytochrome P450 1A, to generate more hydrophilic compounds that are closer in physico-chemical properties to P-gp substrates. To evaluate whether CYP1A metabolites of B[a]P may be transported by P-gp, we also evaluated the distribution of total radioactivity in fish previously treated with 2,3,7,8-tetrachlorodibenzofuran (TCDF), a model CYP1A inducer, as compared to

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previously untreated fish. In contrast to our results with the known P-gp substrate rhB, the distribution of B[a]P and/or its metabolites were not influenced by P-gp-mediated transport in liver, brain, or ovary. We suggest that prior observations of B[a]P active transport in *in vitro* systems were erroneously attributed to P-gp-mediated transport and that B[a]P and its CYP1A metabolites should not be considered P-gp substrates in vertebrates.

The inexpensive and rapid *in vivo* assay used here could be applied to assess the potential of other environmental pollutants and natural products to be transported by P-gp. The evaluation of P-gp expression in natural populations, will help us to better understand the biochemical mechanisms employed to permit animals to survive in environments containing both naturally occurring toxins and anthropogenic contaminants.

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Figure 1. Multiplicity and nomenclature of P-glycoprotein genes
Modified from Borst and Schinkel, 1997)

Organism	Class I Drug transporters		Class II[a] Phosphatidylcholine translocator	Sister of P-gp Bile salt transporter
humans	MDR1		MDR3(MDR2)[b]	
mice	mdr1a(mdr3)[c]	mdr1b(mdr1)	mdr2	
rats	mdr1a	mdr1b	mdr2	spgp
hamsters	pgp1	pgp2	pgp3	
fish	<-------------------fpgp B[d]--------------------------->			fpgp A
marine worm	<-------------------------------------pgp[e]------------------------------------>			

[a]The P-glycoproteins (P-gps) encoded by the human MDR3 and the murine Mdr2 genes encode phosphatidylcholine translocators (Borst and Schinkel, 1996.) In view of the high degree of sequence identity of these two P-gps with the P-gps encoded by the mdr2 and Pgp3 genes of rat and hamsters, it is likely that these are also phosphatidylcholine translocators, but this has not been experimentally verified.

[b]Evidence for sequences corresponding to a second P-gp gene in humans, called MDR2, were first obtained by Roninson and colleagues (Roninson et al., 1986.) A functional and expressed gene was later cloned by Van der Bliek and colleagues (van der Bliek et al., 1988)

[c]The first two murine P-gp gene were discovered and cloned by Ruetz and Gros, (1994), called mdr1 and mdr2. To avoid confusion, the Mdr1a/Mdr1b nomenclature introduced by Hsu and colleagues is used here (Hsu et al., 1989.)

[d]Fpgp B is related to both Class I and II P-gps by sequence, and homology to a specific mammalian isoform by function cannot yet be made (Chan et al., 1992; Cooper, 1996.)

[e]A partial clone of Pgp from *Urechis caupo* has approximately 50% sequence identity to other known P-glycoprotein genes and has functional similarity to class I P-gps (Toomey, 1995.)

For further information on ABC transporter nomenclature refer to two related web sites: www.gene.ucl.ac.uk/users/hester/abc.html or www.med.rug.nl/mdl/tab3.htm

Chapter 2

Review: Multixenobiotic resistance as a cellular

defense mechanism in aquatic organisms

Review

Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms

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Abstract

Multixenobiotic resistance in aquatic organisms exposed to natural toxins or anthropogenic contaminants is a phenomenon analogous to multidrug resistance in mammalian tumor cell lines tolerant of anti-cancer drugs. Multidrug resistance is commonly due to the elevated expression of transmembrane P-glycoproteins (P-gp) which actively transport a wide variety of structurally and functionally diverse compounds. The purpose of this review is to place aquatic ecotoxicological data in context of the larger multidrug resistance field of study. Information on P-glycoproteins structure, mechanism of transport, and substrate specificity gained through traditional mammalian and cell culture models is examined in conjunction with recent work on aquatic species exposed to xenobiotics both in the field and in the laboratory. The physiological function of P-glycoproteins is explored through studies of gene knockout models and expression patterns in normal tissues and tumors. The effect of xenobiotic exposures on P-gp activity and protein titer is examined in wild and captive populations of aquatic invertebrates and vertebrates. Substrate overlap and evidence of co-expression of phase I detoxification enzymes (e.g. cytochromes P450) and P-gp are presented. The role of P-gp chemosensitizers as environmental pollutants and the ecotoxicological consequences of P-gp inhibition are highlighted. The overwhelming evidence suggests that P-glycoproteins provide aquatic organisms with resistance to a wide range of natural and anthropogenic toxins. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Multidrug resistance; Multixenobiotic resistance; P-glycoprotein; Marine; Aquatic; Fish

1. Introduction

Many aquatic species are able to survive in environments which contain high levels of multi-ple anthropogenic pollutants or natural product toxins. This multixenobiotic resistance (MXR) phenomenon is similar to multidrug resistance (MDR) first observed in tumor cell lines resistant to anti-cancer drugs. Overexpression of a 170 kDa transmembrane P-glycoprotein (P-gp) was found to prevent the accumulation of cytotoxic drugs in

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resistant cells. MDR is of clinical importance because many human tumors have inherent or acquired P-gp-mediated drug resistance and do not respond to chemotherapy. P-gp is found endogenously in specialized epithelial tissues involved in secretion and excretion such as the mammalian gut, liver, and, kidney, as well as on endothelial cells of capillary blood vessels at the blood-brain barrier.

P-gp acts as an energy-dependent pump to translocate a wide variety of structurally and functionally diverse substrates. These compounds tend to be moderately hydrophobic, planar, natural products which are often substrates for or metabolites of detoxification enzymes such as cytochromes P450 (CYPs.) P-gps prevent the cellular accumulation of endogenous metabolites, phospholipids, and xenobiotics in exposed animals and cell cultures.

P-gp-like proteins have been described in a variety of aquatic organisms including sponges, mussels, oysters, clams, worms, and fish. Both natural products and anthropogenic contaminants found in the aquatic environment appear to be substrates and inducers of the multixenobiotic resistance transporter in aquatic organisms. These observations suggest that in addition to normal cell function, P-gp activity may contribute to the relative hardiness of some aquatic species exposed to xenobiotics. In addition to well characterized detoxification systems (phase I, II, III enzymes, heat shock proteins, etc.), the induction of a multixenobiotic defense mechanism in organisms living in polluted environments may explain why contaminant spills cause more severe adverse effects at pristine sites than in already polluted areas.

Although P-gps have been intensively studied in relation to cancer biology using highly selected drug resistant cell lines and chemotherapeutic agents, less is known regarding multixenobiotic resistance in wild populations of organisms and there is similarly little information on environmentally relevant P-gp substrates. Several excellent reviews have been previously published on P-glycoprotein-mediated multixenobiotic resistance in aquatic organisms including the first overview of the field (Kurelec, 1992), followed by articles describing the results of inhibiting MXR (Kurelec, 1995a,b), chemosensitizers as a hazardous new class of pollutants (Kurelec, 1997), and MXR as a first line of defense against toxins (Epel, 1998). The purpose of this review is to place ecotoxicological data in context of the larger multidrug resistance field of study. Information on P-glycoprotein structure, mechanism of transport, and substrate specificity gained through traditional mammalian and cell culture models will be examined in conjunction with recent work on wild aquatic organisms exposed to xenobiotics both in the field and in the laboratory.

2. Background: multidrug resistance

Multidrug resistance is a phenomenon which was first observed in tumor cell lines selected for resistance to a single agent which developed simultaneous resistance to a wide variety of structurally and functionally unrelated antineoplastic drugs (Kessel et al., 1968; Biedler et al., 1975; Nielsen and Skovsgaard, 1992). Human cancer patients were observed through the course of chemotherapy to become resistant to not only the initial drug but also a host of other antiproliferative agents to which they had not been previously exposed. Juliano and Ling discovered that a 170 kDa P-glycoprotein (P-gp) was responsible for the resistance of some tumor cells to anti-cancer drugs (Juliano and Ling, 1976). This phosphorylated and glycosylated P-glycoprotein is localized in the membrane of resistant cells. P-gp consists of a 1280 amino acid transmembrane dimer which acts as an energy dependent efflux pump to prevent the accumulation of drugs within the cell. Each half of the protein consists of six transmembrane regions, a cytoplasmic domain and an ATP binding site (Chen et al., 1986). Recent reviews have detailed drug resistance (Skovsgaard et al., 1994; Ambudkar et al., 1999); P-gp structure and evolutionary homologies (Bosch and Croop, 1998); pharmacology of MDR chemosensitizers (Ford and Hait, 1990); assays to quantify P-gp (van der Heyden et al., 1995); P-gp function (Borst and Schinkel, 1997; Schinkel, 1998).

P-glycoproteins undergo considerable post-translational modification including glycosylation and phosphorylation. Three carbohydrate side chains are located in the first extracellular loop of MDR1. The absence of N-glycosylation sites reduces drug transport efficiency although substrate specificity is not altered (Schinkel et al., 1993). This observation suggests that N-glycosylation contributes to proper routing of the protein to the plasma membrane or stability of P-glycoprotein, but not directly to drug transport. P-glycoproteins are phosphorylated at many threonines and serines (Staats et al., 1990) but how phosphorylation relates to function is currently unclear. P-gp phosphorylation by protein kinase A and C have been observed to slightly enhance ATPase and substrate transport activities (Shapiro and Ling, 1995). However drug transport activity was not altered in P-gps whose Ser/Thr were replaced by the unphosphorylatable Ala or by Asp (whose negative charge mimics that of phosphorylated Ser/Thr) (Germann et al., 1996; Goodfellow et al., 1996).

Highly conserved MDR genes have been described in diverse taxa including rodents (Ng et al., 1989), chicken (Edelmann, 1999) fruit fly (Dressen et al., 1988), nematode worm (Broeks et al., 1995), plants (Dudler and Hertig, 1992; Wang et al., 1996; Sidler et al., 1998; Davies et al., 1999), archaebacteria and bacteria (Felmlee et al., 1985), and several aquatic organisms including winter flounder *Pleuronectes americanus* (Chan et al., 1992), killifish (also known as mummichog) *Fundulus heteroclitus* (Cooper et al., 1998) and a marine worm *Urechis caupo* (Toomey, 1995). Putative P-glycoproteins have been reported in midge larvae (*Chironomus riparius*) and tobacco hornworm (*Manduca sexta*) and sheep nematode parasites (*Haemonchus contortus*) which are resistant to agricultural pesticides (Lanning et al., 1996; Podsiadlowski et al., 1998; Xu et al., 1998). The wide distribution of P-gp genes across phylogenetic taxa suggests that multixenobiotic resistance proteins may be common to all organisms and may transport endogenous substrates in addition to foreign natural products. This review focuses on the role of P-gps in contributing to MXR in wild populations for which data is most abundant for aquatic organisms.

Two classes of mammalian MDR genes which code for functionally different P-glycoproteins have been well characterized. Refer to Fig. 1 for multiplicity and nomenclature of P-gp genes across taxa. Class I genes (human MDR1 and mdr1a and mdr1b in rats) confer constitutive and inducible drug and xenobiotic resistance while class II genes encode a constitutive, apparently uninducible phosphatidylcholine translocator (van Helvoort et al., 1996). A recently described, related 160 kDa P-glycoprotein, dubbed Sister of P-gp or spgp, is localized to the bile canalicular microvilli and subcanalicular vesicles of hepatocytes (Childs et al., 1995; Gerloff et al., 1998) with lower expression detected in the brain gray cortex, small- and large-gut mucosa (Török et al., 1999). Spgp is believed to be the major canalicular bile salt export pump of the mammalian liver which transports primary bile salts such as taurocholate (Gerloff et al., 1998). Spgp-related proteins have been indicated in humans, rats, mice, chicken, turtle, winter flounder and *F. heteroclitus* (Childs et al., 1995; Cooper et al., 1998; Gerloff et al., 1998).

Two fish P-glycoprotein (fpgp A and B) genes have been partially cloned in winter flounder and killifish (Chan et al., 1992; Cooper et al., 1998.) Fpgp A corresponds to spgp while fpgp B is related to both class I and class II mammalian P-glycoproteins (Cooper, 1996; Childs, 1998 personal communication). The partially sequenced *U. caupo* P-glycoprotein has approximately 50% sequence homology to mammalian class I, class II, and spgp (Toomey, 1995). The anti-P-glycoprotein C219 monoclonal antibody recognizes an internal, highly conserved amino acid sequence near the nucleotide binding domain common to all P-glycoprotein isoforms whose sequence is known (Kartner et al., 1985; Endicott and Ling, 1989). Studies of aquatic invertebrates have identified P-glycoproteins with mAb C219 and have demonstrated functional homology to the xenobiotic resistance transporter, but this information is insufficient to definitively classify these P-glycoproteins by mammalian nomenclature. Furthermore, many of the mammalian studies cited in this review do not distinguish between P-glycoprotein isoforms.

For the purposes of this review, the term *P-gly-coprotein* and the abbreviation P-gp will be used to refer to all three known P-glycoprotein types: class I: drug transporter; class II: phospholipid flippase; spgp: bile salt transporter. P-glyco-proteins will be referred to by specific isoform or class where data is available. The term *multixeno-biotic resistance transporter* will refer to the multiple P-glycoproteins identified in aquatic organisms and associated with functional resistance to xenobiotics. The term *P-glycoprotein substrate* will refer to a compound which might be transported by any of the three isoform types. Where data is available, specificity of substrates to distinct isoforms will be noted.

P-gps are members of the ABC (ATP-binding cassette) superfamily of structurally similar transmembrane transport proteins present in eubacteria, eukaryotes, and recently described in Archaea (Jovell et al., 1996; Xavier et al., 1996; Koonin et al., 1997, recently reviewed by Ambudkar et al., 1999). In eukaryotes, ABC transporters

function to remove molecules from the cytoplasm and export them either through the cell cytoplasmic membrane or through organelle membranes (Saurin et al., 1999). Prokaryotes have two types of ABCs involved in either export or uptake of molecules (Saurin et al., 1999). ABC transporters include hCFTR, the human cystic fibrosis transmembrane conductance regulator (Higgins et al., 1986), *pfmdr* chloroquine resistance in *Plasmodium falciparum* the organism responsible for malaria (Foote et al., 1989), *STE6* lipopeptide transporter in *Saccharomyces cerevisiae* (McGrath and Varshavsky, 1989), human LRP the lung resistance protein (Scheper et al., 1993), and mammalian MRP the multidrug-resistance-associated protein (Cole et al., 1992).

LRP and MRP provide MDR in some tumor cell lines which do not overexpress P-gp. The 110 kDa LRP is the major cytoplasmic vault protein involved in nuclear-cytoplasmic transport (Scheffer et al., 1995). MRPs are 190 kDa glycoproteins with three multispanning transmembrane domains

Organism	Class I Drug transporters		Class II[a] Phosphatidylcholine translocator	Sister of P-gp Bile salt transporter
humans	MDR1		MDR3(MDR2)[b]	
mice	mdr1a(mdr3)[c]	mdr1b(mdr1)	mdr2	
rats	mdr1a	mdr1b	mdr2	spgp
hamsters	pgp1	pgp2	pgp3	
fish	<---------------fpgp B[d]---------------------->			fpgp A
marine worm	<--------------------------------pgp[e]--------------------------------->			

[a]The P-glycoproteins (P-gps) encoded by the human MDR3 and the murine Mdr2 genes encode phosphatidylcholine translocators (Borst and Schinkel, 1996.) In view of the high degree of sequence identity of these two P-gps with the P-gps encoded by the mdr2 and Pgp3 genes of rat and hamsters, it is likely that these are also phosphatidylcholine translocators, but this has not been experimentally verified.

[b]Evidence for sequences corresponding to a second P-gp gene in humans, initially dubbed MDR2, were first obtained by by Roninson and colleagues (Roninson et al., 1986.) A functional and expressed gene was later cloned by Van der Bliek and colleagues (van der Bliek et al., 1988)

[c]The first two murine P-gp gene cloned were called mdr1 and mdr2 (Ruetz and Gros, 1994.) To avoid confusion, the Mdr1a/Mdr1b nomenclature introduced by Hsu and colleagues is used here (Hsu et al., 1989.)

[d]Fpgp B is related to both Class I and II P-gps by sequence, and homology to a specific mammalian isoform by function cannot yet be made (Chan et al., 1992; Cooper, 1996.)

[e]A partial clone of Pgp from *Urechis caupo* has approximately 50% sequence identity to other known P-glycoprotein genes and has functional similarity to class I P-gps (Toomey, 1995.)

For further information on ABC transporter nomenclature refer to two related web sites: www.gene.ucl.ac.uk and www.med.rug.nl/mdl/tab3.htm

Fig. 1. Multiplicity and nomenclature of P-glycoprotein genes (modified from Borst and Schinkel, 1997).

compared to two in MDR1 P-glycoprotein and these protein families share between 14–25% amino acid identity (Cole et al., 1992; Keppler and König, 1997). The human MRP family contains at least six members which have on average 50% amino acid identity between them (Keppler and König, 1997; König et al., 1999): MRP1 gene encodes multidrug-resistance-associated protein in extrahepatic tissues (Cole et al., 1992); MRP2 or cMOAT encodes the canalicular multispecific organic anion transporter found in liver and kidney (Buchler et al., 1996; Schaub et al., 1997); basolateral MRP3 (BLMRP) is expressed in the basolateral membrane of hepatocytes, colon, pancreas and kidney (König et al., 1999); MPR6 is expressed in liver and kidney but its function is unknown (Kool et al., 1999); and two additional homologues, MRP4 and MRP5 whose distribution and function are unknown (Kool et al., 1997). MRP1 and MRP2 are responsible for export of amphiphilic anions and anionic conjugates formed via metabolism by phase II enzymes (Cole et al., 1994; Zaman et al., 1995), for example, cholestatic glucuronidated steroids (Loe et al., 1996) and leukotriene C4 glutathione conjugate, an arachidonic acid metabolite important for regulation of blood pressure (Lautier et al., 1996). MRPs are believed to be the previously described GS-X pump, a member of the phase III drug metabolizing system (Ishikawa, 1992). MRP-mediated transport may both complement and supplement P-gp efflux due to some substrate overlap between the two systems (Loe et al., 1998; Robson et al., 1998).

3. Mechanism of transport

The mechanism by which P-glycoprotein transports xenobiotics is unknown. Initially, some groups presented evidence that P-gp functions as both an ATP channel (Abraham et al., 1993) and a swelling-activated Cl- channel (Valverde et al., 1992; Wine and Luckie, 1996). These claims of channel activity have been refuted (Boyum and Guidotti, 1997), however, data suggests that P-gp may regulate the activity of other Cl-channels in some cell types and be involved in osmotic control

(Higgins, 1995). The classic pump model of membrane transporters is insufficient to explain all aspects of P-gp transport (Sharom, 1997). In the classical model, the membrane transporter forms an aqueous pore which undergoes a conformational change in order to move a hydrophilic substrate from the aqueous phase cytosol to the aqueous phase extracellular space. Although this model may not be rejected outright, several modifications are required since the substrates are generally hydrophobic and transporter-substrate binding occurs in the lipid phase of the membrane. Three models have been proposed to explain P-gp-mediated transport: hydrophobic vacuum cleaner model (Higgins and Gottesman, 1992), flippase (Higgins and Gottesman, 1992), and extrusion from the inner leaflet (Eytan and Kuchel, 1999).

Higgins and Gottesman proposed the *hydrophobic vacuum cleaner* model in which drugs are transported from the plasma membrane to the extracellular medium (Higgins and Gottesman, 1992). The model presents a two-tier recognition process: the primary determinant of substrate specificity is a compound's ability to intercalate into the membrane (hydrophobicity) and the following interaction of the compound with a relatively nonselective binding site on P-gp would be of secondary importance. But this model too was not entirely satisfactory in part because P-gp was shown to specifically bind to substrates with different specificities (Liu and Sharom, 1996).

In response, a second model was proposed that postulates P-gp flips drugs from the inner to the outer leaflet of the plasma membrane against an intramembrane concentration gradient and then diffuse out of the cell (Higgins and Gottesman, 1992). The term *flippase* was originally coined to describe membrane enzymes that translocate phospholipids. Although the lateral mobility of phospholipids is high, movement between leaflets is low because the polar head group of the phospholipid has difficulty passing through the hydrophobic interior of the membrane. Flippases speed up this process. Support for this model was provided by Smit et al.'s discovery that mouse canalicular mdr2 facilitates the normal transport of phosphatidylcholine from the hepatocyte into

the bile (Smit et al., 1993). Recent work with pig kidney cells transfected with class I and class II mdr cDNA constructs revealed that both isoforms can translocate short-chain fluorescent phospholipid analogs (van Helvoort et al., 1996). MDR1 is presumed to transport molecules which intercalate and introduce discontinuities into the bilayer, such as amphipathic drugs, by the same flippase mechanism (Higgins and Gottesman, 1992).

A recently proposed third model, which combines elements of the two previous models, postulates that P-gp extracts substrates from the inner leaflet and extrudes them into the extracellular domain (Eytan and Kuchel, 1999). In this model, first the hydrophobic, cationic P-gp substrates in the extracellular medium readily bind to the negatively charged outer leaflet of the lipid bilayer. Thus the direct source of drug taken up into cells is the high concentration cellular bound drug pool rather than the low concentration drug pool in the medium. These compounds then move between the outer and inner bilayer via a slow flip-flop mechanism rather than diffusion down a concentration gradient within the lipid bilayer. Drugs in the inner leaflet are in equilibrium with a drug pool in the cytoplasm which bind to intracellular molecular sinks such as DNA. Substrates in the inner leaflet bind to P-gp and are actively flipped out to the extracellular medium (Eytan and Kuchel, 1999). The ATP stochiometry of this process is 1:1 with the two ATP-binding sites active but alternating in catalysis (Higgins, 1992; Garrigos et al., 1993; Urbatsch et al., 1995). Because P-gp substrates flip-flop slowly across the membrane on their way to the cytoplasm, fast P-gp-mediated transport can efficiently remove them from the cell. In contrast, chemosensitizers diffuse across the membrane quickly and reenter the cells faster than P-gp can remove them, thus rending their extrusion futile (Eytan and Kuchel, 1999).

The wide substrate specificity of P-gp is an intriguing problem and much work has been directed toward characterizing the binding site(s). Recently, Shapiro and Ling presented evidence that P-glycoprotein contains at least two distinct substrate binding and transport sites and that these sites interact in a positively cooperative

fashion (Shapiro and Ling, 1997). They propose that one site dubbed R (for the substrate rhodamine 123 and anthracyclenes) binds one set of compounds and the other site H (for the substrate Hoechst 33342 and colchicine) binds a second set (Shapiro and Ling, 1997, 1998). Each R substrate stimulates the P-glycoprotein-mediated transport of an H substrate and vice versa. Two R substrates would compete for the R site and inhibit binding and transport of the other. Competitive inhibition would also be observed between two H substrates or between substrates which can bind to both R or H sites. A third drug-binding site (for the substrates prazosin and progesterone) on the P-glycoprotein exerts a positive allosteric effect on drug transport by the H and R sites but is not thought to be capable of drug transport itself (Shapiro et al., 1999). Shapiro and Ling's three-site model is consistent with previous observations of both competitive and non-competitive interactions between P-gp substrates.

4. P-glycoprotein substrates

The basis for the extremely broad substrate specificity of P-gp has not yet been elucidated. P-gp substrates differ in cytologic target, chemical structures, and properties (Endicott and Ling, 1989). The only obvious physical similarities substrates share are being moderately hydrophobic, amphipathic (i.e. somewhat soluble in both lipid and water), low molecular weight, planar molecules with a basic nitrogen atom, cationic or neutral but never anionic, and natural products (Gottesman and Pastan, 1988; Endicott and Ling, 1989; Pearce et al., 1990; Gottesman et al., 1994). P-gp substrate chemotherapeutic drugs include colchicine, Vinca alkaloids (e.g. vinblastine, vincristine), actinomycin D, taxol, epipodophyllotoxins (e.g. etoposide), and anthracyclines (e.g. doxorubicin). Other natural product compounds transported by P-gp include calcium channel blockers (e.g. verapamil and dihydropyridines), antiarrhythmics (e.g. quinidine), antihypertensives (e.g. reserpine), steroids (e.g. cortisol, dexamethasone, aldosterone), and antiparasitics (e.g. quinine, ivermectin) to list a small number. Sev-

eral groups have reported P-gp structure-activity models to help predict which drugs will interact with P-gp (Zamora et al., 1988; Bain et al., 1997; Klopman et al., 1997; Etievant et al., 1998; Seelig, 1998).

Natural product screens have discovered numerous P-gp substrates derived from aquatic organisms including okadaic acid, the causative agent in human diarrhetic shellfish poisoning derived from the marine alga *Dinophysis* spp. (Suganuma et al., 1988); calyculin A, a potent tumor promoter and protein phosphatase inhibitor isolated from the marine alga *Acetabularia* sp. (Chambers et al., 1993); patellamide D a cyclic octapeptide isolated from the marine tunicate, *Lissaclinum patella* (Williams and Jacobs, 1993); dolastatins, small peptides isolated from the marine sea hare *Dolabella auricularia* (Aherne et al., 1996); and lamellarins, polyaromatic alkaloids isolated from tunicates (genus *Didemnum*) (Quesada et al., 1996).

Non-cytotoxic compounds such as fluorescent rhodamine dye and the calcium channel blocker verapamil, a cardiovascular medication, have been used respectively as a model substrate and model competitive inhibitor (Neyfakh, 1988; Ford and Hait, 1990). The competitive inhibition of rhodamine efflux in a model vesicle or cellular system by the addition of verapamil is considered evidence of P-gp activity. Furthermore, the ability of a chemical to inhibit rhodamine transport in an assay system is considered evidence of a potential P-gp substrate or inhibitor.

Verapamil which can competitively inhibit the transport of P-gp substrates functions as a *chemosensitizer* or *modulator* of multidrug/xenobiotic resistance. Chemosensitizers tend to be more lipophilic than other substrates and once extruded by P-gp, diffuse back into the cell before retransport (Sharom, 1997). Drugs referred to as *substrates* are found experimentally to diffuse relatively slowly across membranes on the order of minutes to hours compared to chemosensitizers which traverse bilayers too quickly to measure (Eytan et al., 1996). Clinicians have co-administered verapamil with chemotherapeutic drugs in hopes of eradicating MDR tumors in cancer patients. Unfortunately, the chemosensitizer dose required to inhibit P-gp provokes cardiovascular side effects. In phase II clinical trials, second generation chemosensitizers with limited side effects have proved promising for a subset of patients (Kornek et al., 1995; Lehnert et al., 1998). Care must be taken in designing such chemosensitizer treatments as mdr knockout mice studies suggest that lack of functional P-glycoprotein can altered drug pharmacokinetics in ways which could lead to accumulation of drugs in non-target organs such as the brain or testes (Mayer et al., 1997; Hendrikse et al., 1998).

Some modulators are not in fact P-gp substrates but influence P-gp indirectly to disrupt transport of normal substrates. For example, staurosporine, a protein kinase C inhibitor, has been observed to inhibit P-gp substrate transport presumably by disrupting P-gp phosphorylation (Kurelec, 1995b). ATPase inhibitors, membrane fluidizers and permeabilizers may also act as modulators (Sharom, 1997). Molecules which have the ability to bind to P-gp but are not transported can also disrupt efflux of model P-gp substrates. Using an MDR1 transfected cell line, non-substrate pesticides were screened for their ability to bind to P-gp and inhibit the cellular efflux of the P-gp substrate doxorubicin (Bain and LeBlanc, 1996). Organophosphorus and organochlorine pesticides had the greatest inhibitory effect. Optimum binding occurred with inhibitor compounds having a cyclic structure within the molecule, a low Kow value (3.6–4.5), and a molecular weight of 391–490 kDa. Environmental exposure of wild populations to these pesticide inhibitors could result in increased sensitivity to natural product toxins which the organisms would be normally resistant to when P-gp activity is unhindered. The concept of xenobiotic chemosensitizers as a new class of environmental pollutants, an idea originally proposed by Kurelec (1995a) will be discussed later in this review in the section entitled *Chemosensitizers as environmental pollutants*.

5. P-glycoprotein expression in normal tissues and tumors

P-glycoprotein expression is detected in three distinct subsets of normal human tissue involved

in secretion, absorption or a barrier function, and in some tumors derived from these tissues: a subset of columnar epithelial cells, endothelial cells of capillary beds in specific anatomic locations, and placental trophoblasts. (The trophoblast is a thin layer of ectoderm that constitutes the wall of a mammalian blastula and is important for the nutrition and implantation of the embryo.) Elevated P-gp expression in tumors prior to treatment is a poor prognosticator of chemotherapy success (van der Heyden et al., 1995).

Human MDR1 has been detected at high levels in liver, kidney, small bowel, colon, pancreas, adrenal cortex, placenta, testis-brain barrier, and blood-brain barrier (Fojo et al., 1987; Sugawara et al., 1988; Cordon-Cardo et al., 1990; Sugawara, 1990). MDR1 P-gp transports drugs and xenobiotics that are unmodified by phase II cellular metabolizing enzymes. Evidence suggests that MDR1 P-gp in the adrenal cortex is involved in transport of steroid hormones particularly glucocorticoids (Naito et al., 1989; Ueda et al., 1992; Wolf and Horwitz, 1992). The MDR2 encoded P-gp is not involved in the transport of hydrophobic anticancer drugs. This isoform is highly expressed in the canalicular membranes of hepatocytes (Smit et al., 1994) and has been detected at low levels in the spleen, B cells, heart and muscle (Buschman et al., 1992). Canalicular MDR2 is involved in the transport of phospholipids into bile (Smit et al., 1993; van Helvoort et al., 1996). Sister of P-glycoprotein is localized in the canalicular microvilli and to subcanalicular vesicles of the hepatocytes and functions as the major canalicular bile salt export pump of mammalian liver (Childs et al., 1995; Gerloff et al., 1998). Spgp expression has also been detected in the brain gray cortex, small and large gut mucosa by RT-PCR (Török et al., 1999). P-glycoprotein expression has been detected in aquatic organisms by two approaches: immunochemistry and activity assays. Each will be addressed in turn.

5.1. Immunochemical evidence for P-glycoprotein expression in aquatic organisms

Antibodies against mammalian P-glycoprotein epitopes cross react in aquatic organisms in similar tissue types as mammals. Although several antibodies demonstrate cross reactivity, the most successful and widely used antibody is C219. As previously mentioned, the C219 mAb recognizes a highly conserved linear epitope located near the nucleotide binding domain and identifies all known P-glycoproteins (Kartner et al., 1985). Since all three human P-gps are approximately 170 kDa they can only be distinguished by isoform specific antibodies which have not been evaluated for use in aquatic organisms. Since C219 cannot distinguish between P-gp isoforms and there is limited information on intraspecific diversity of P-gp isoforms in aquatic organisms, the following section will describe expression of total P-gp expression and function in aquatic organism.

Proteins which are immunologically related to the mammalian P-glycoproteins have been identified by Western blot in tissues involved in absorption, secretion and a barrier function in several aquatic invertebrate species: 135 kDa molecule in the clam Corbicula fluminea (Waldmann et al., 1995); > 200 kDa proteins in oyster gill Crassostrea gigas and C. virginica (Minier et al., 1993; Keppler, 1997); 170 kDa protein (plus faint signal at > 200 kDa) in gills of mussels Mytilus californianus, M. galloprovincialis, and M. edulis (Cornwall et al., 1995; Galgani et al., 1995; Kornek et al., 1995; Minier and Moore, 1996a,b); 140 kDa protein in the marine snail Monodonta turbinata (Kurelec et al., 1995b); 140 kDa protein in the egg membrane of U. caupo and a 110 kDa protein in the adult worm anterior digestive tract and epidermal tissue (Toomey and Epel, 1993); and 125–130 kDa proteins in the cell membrane of marine sponges Geodia cydonium, Verongia aerophoba, and Suberits domuncula (Kurelec et al., 1992; Muller et al., 1996.) Refer to Fig. 2 for a list of aquatic organisms and the tissues in which C219 reactive proteins have been detected. The range of molecular weights detected for P-gps in different aquatic species is not inconsistent with mammalian studies. Reported mammalian P-gps are heavily glycosylated and are expected to produce a diffuse band on Western blots as a results

Common name		Scientific name	Tissues	Reference
Clam	Asiatic clam	Corbicula fluminea	gill	Waldmann, 1995
	freshwater	Dreissena polymorpha	gill	Smital and Kurelec, 1997
Crab	Shore crab	Carcinus maenas	hepatopancreas	Köhler et al., 1998c
Fish	Slender cockscomb blenny	Anoplarchus insignis	liver	Bard et al., 1998
	High cockscomb blenny	Anoplarchus purpurescens	liver	Bard et al., 1998
	Sheeps head minnow	Cyprinodon variegatus	liver, exocrine pancreas, intestine, kidney	Hemmer et al., 1998
	Carp	Cyprinus carpio	liver	Kurelec, 1995b
	Mummichog/Killifish	Fundulus heteroclitus	liver, intestine, kidney	Cooper, 1996 Schramm et al., 1995
			MRP in kidney	* Masereeuw et al., 1996
			opercular epithelium	Karnaky et al., 1993
	Channel catfish	Ictalurus punctatus	intestine, liver	Doi et al., 1999
	Golden ide	Leuciscus idus melanotus	liver	Kurelec, 1992c
	Dab	Limanda limanda	liver	Smital and Kurelec, 1998b
	Sea mullet	Mugil cephalus	liver	Smital and Kurelec, 1998b
	Winter flounder	Pleuronectes americanus	liver	Chan et al., 1992
	Black prickleback	Xiphister atropurpureus	liver	unpublished data from our laboratory
	Rock prickleback	Xiphister mucosus	liver	unpublished data from our laboratory
	European flounder	Platichthys flesus L.	liver	Köhler et al., 1998a
	Guppy	Poecilia reticulata	liver, exocrine pancreas, intestine, kidney, branchial gas gland, gill	Hemmer et al., 1995
	Dogfish shark	Squalus acanthias	MRP in rectal gland	* Miller et al., 1998a
	Eelpout	Thermarces cerberus	liver	unpublished data from our laboratory
Mussel	Swan mussel	Anodonta cygnea	gill	Kurelec, 1992c
	California mussel	Mytilus californianus	gill	Cornwall, 1995
	Blue mussel	Mytilus edulis	gill	Minier, 1993
			blood cells	Minier, 1996
			embryo	McFadzen et al., 1999
	Bay mussel	Mytilus galloprovincialis	gill	Kurelec, 1991
	Brown mussel	Perna perna	gill	Grimm et al., 1999
Oyster	Pacific oyster	Crassostrea gigas	gill	Minier, 1993
	American oyster	Crassostrea virginica	gill, embryo	Keppler, 1997
Shrimp	Grass shrimp	Palaemonetes pugio	intestine, embryo	Scott et al., 1999
Snail		Monodonta turbinata	gill	Kurelec, 1995a
		Paludina vivipara	gill	Smital and Kurelec, 1998b
Sponge		Geodia cydonium	sponge cells	Kurelec, 1992a
		Suberites domuncula	sponge cells	Muller, 1996
		Tethya aurantium	sponge cells	Kurelec, 1992b
		Verongia aerophoba	sponge cells	Kurelec, 1992a
Toad	African clawed toad	Xenopus laevis	intestine, embryos	Zucker et al., 1997
Worm		Urechis caupo	intestine, epidermis, embryos	Toomey and Epel, 1993

Fig. 2. Multixenobiotic resistance in marine and freshwater organisms was detected in noted tissues by immunoreactivity with mammalian P-gp antibodies, from P-gp-like transport activity of model substrates, or genetic data. P-gp hepatic expression in vertebrates is localized to the bile canaliculi. Evidence for presence of MRP-like proteins denoted by an asterick (*).

of microheterogeneity in glycosylation (Richert et al., 1988). Depending on the state of glycosylation, mammalian P-gps can range from molecular weight of 130–170 kDa (Kartner et al., 1985).

Protocols for Western blot and immunohistochemical detection of P-gp are available for analysis of fish tissues (Hemmer et al., 1995; Cooper et al., 1999). For immunohistochemical analysis, fixation and staining conditions can alter antibody reactivity dramatically thus only samples which have undergone a standardized immunochemical protocol may be compared. A difference

in relative immunostaining intensity with a variety of anti-P-gp antibodies applied to fish tissues was observed depending on histological fixative (Lillie's > Dietrich's > Bouin's) (Hemmer et al., 1998). The effectiveness of formalin, a fixative which is commonly used in the clinical setting, for the majority of mammalian P-gp studies was not evaluated in this study. Formalin fixation may attenuate P-gp signal due to substantial cross-linking and thus for maximum signal, samples should be fixed for as short a period of time as possible, or alternatively frozen tissue sections can

be used (Cordon-Cardo et al., 1990; Stieger, 1999 personal communication).

A 170 kDa protein cross-reacts with C219 in liver of several species of fish including, *F. heteroclitus* and *Anoplarchus* sp. (Bard et al., 1998; Cooper et al., 1999). In guppy (*Poecilia reticulata*), P-gp expression has been reported in bile canaliculi, gill chondrocytes, pseudobranch, kidney renal tubules, pancreatic exocrine tissue, gas gland, and intestinal epithelium (Hemmer et al., 1995). In channel catfish, C219 detectable protein is highly expressed along the luminal mucosa of the distal intestine with no expression detected in the proximal intestine (Kleinow et al., 1999).

The frog P-gp homolog, Xe-mdr, is localized to the secretory epithelium of the intestinal lumen (Castilo et al., 1995.) Developmental studies of *Xenopus* determined that Xe-mdr is first detected in the early tadpole (stage 40), prior to onset of feeding (Zucker et al., 1997). Stage 40 is characterized by the mouth breaking through the oral plate and the separation of the tracheal cavity from gastro-duodenal cavity. Xe-mdr expression increases during intestinal development a trend consistent with P-gp's presumed role in intestinal transport of dietary xenobiotics (Zucker et al., 1997).

P-gp in crab (*Carcinus maenas*) hepatopancreas is localized to the microvilli of epithelial cells and to lysosomal membranes within F/B stage cells in the tubules (Köhler et al., 1998c). P-gp expression in the microvilli may form a first line of defense against uptake of toxins and as sites of intensive elimination. F/B is a lysosome-rich transitional stage between fibrillar (F) cells and blister (B) cells in the hepatopancreas tubule. P-gp may act to transport xenobiotics from F/B cell cytoplasm into lysosomes for later elimination in the late B cell stage. In aging B cells, P-gp expression decreases in conjunction with the fusion of lysosomal compartments to form one large vacuole and subsequent expulsion into the digestive lumen. Concentration of model P-gp substrates (rhodamine B) in lysosomes has previously been observed in blood cells of mussels (*M. edulis*) to increase after inhibition of P-gp transport by the inhibitor verapamil (Minier and Moore, 1996a,b). F/B cells may function to facilitate the accumula-

tion, digestion, and elimination of toxic substances (Köhler et al., 1998c).

5.2. P-glycoprotein expression in fish tumors

The immunohistochemical detection of P-glycoproteins in liver tumors has recently been investigated in three fish species: European flounder, killifish, and winter flounder. In the first study, hepatocellular carcinomas were detected in 40% of European flounders (*Platichthys flesus*) collected from a site contaminated with aromatic hydrocarbon (Köhler et al., 1998b). P-gp was weakly expressed in the bile canaliculi of livers of healthy flounder. In non-neoplastic degenerated liver and extrafocal liver parenchyma surrounding tumors, P-gp was patchily distributed and levels were similar to that to healthy fish. The greatest P-gp titer was observed in satellites and rims of carcinomas which were invasively expanding into extrafocal liver tissue. This pattern of P-gp expression in hepatocellular carcinoma of contaminated fish resembles the classical mammalian multidrug resistance phenotype (Köhler et al., 1998b).

In the second study, a population of killifish living in a creosote-contaminated estuary was resistant to acute toxicity but developed hepatocellular carcinomas (Williams, 1994; Vogelbein et al., 1996). P-gp was elevated two- to threefold in both liver and advanced stage hepatocellular carcinomas of resistant fish compared to a reference population (Cooper et al., 1999). Furthermore, the majority of the hepatocellular carcinomas examined showed clear elevation of P-gp within tumors compared with adjacent parenchyma. Many carcinomas had delocalization and loss of polarity of P-gp expression with staining observed in the cytoplasm and along the entire plasma membrane rather than localized to the bile canaliculi as in healthy fish. These results suggest that elevated P-gp expression in fish hepatocellular carcinomas may be related to tumor progression rather than selection of resistant cells early in carcinogenesis (Cooper et al., 1999).

In the final study, P-gp expression was investigated in winter flounders (*P. americanus*) from Boston Harbor, MA which are highly contaminated with PAHs, HAHs, and heavy metals

(Moore and Stegeman, 1994; Bard et al., 1997). Cholangiocellular neoplasms were evident in 10% of flounders examined (Moore and Stegeman, 1994). The pattern of expression of P-glycoprotein was unusual and novel (Bard et al., 1997). Unexpectedly, only 13% of the cholangiocellular carcinoma samples showed detectable expression of P-gp. In contrast, 80% of the tumor-bearing fish from Boston Harbor had elevated P-gp in the bile canaliculi of non-tumorous tissue. P-gp was not detected in non-tumor bearing fish. The elevated P-gp expression in normal tissue of tumor-bearing animals compared to healthy fish may be due to the influence of the tumor on the surrounding parenchyma. Other workers have found that increased P-gp levels can be induced in non-tumorous tissue in response to liver injury or partial hepatectomy (Fairchild et al., 1987). The tumor may signal normal tissue to increase P-gp in a similar way that liver cells surrounding injured liver tissue overexpress P-gp as a putative general defense mechanism (Bard et al., 1997).

5.3. Detection of xenobiotic transport in aquatic organisms

In addition to immunohistochemical evidence, P-gp expression has been detected by sensitive competitive inhibition substrate binding assays in multiple aquatic organisms and tissues. Membrane vesicles prepared from the gill, mantle, and digestive gland of a marine mussel, *Mytilus galloprovincialis*, bound acetylaminofluorene, an MDR1 P-glycoprotein substrate, in a saturable and verapamil-sensitive manner characteristic of drug-resistant cell lines (Kurelec and Pivcevic, 1991). Verapamil also suppresses the binding of the P-gp substrate [^{3}H]-vincristine to membrane vesicles prepared from freshwater clams (*Corbicula fluminea*) and marine sponges (*Suberits domuncula* and *Tethya aurantium*) (Kurelec and Pivcevic, 1992; Waldmann et al., 1995; Muller et al., 1996).

A second method used to determine P-gp activity is by competitive transport assays in which there is decreased efflux of a radioactive or fluorescence labeled P-gp substrate in the presence of a P-gp inhibitor leading to accumulation of labeled substrate in examined cells (Cornwall et al., 1995). Individual cells of the sponge *Suberites domuncula* incubated with either [^{3}H]-vincristine or the fluorescent dye calcein-AM were found to accumulate approximately twofold more P-gp substrate in the presence of verapamil (Muller et al., 1996). P-gp-specific transport of the fluorescent P-gp substrate daunorubicin was detected across intestine and embryonic cell coat of the grass shrimp (*Palaemonetes pugio*) (Finley et al., 1998). Transport was inhibited by verapamil and endosulfan, a common estuarine contaminant previously shown to be a P-gp substrate (Bain and LeBlanc, 1996). mAb C494 was used to localize P-gp to the luminal epithelial cells of intestine and outer epidermis under the exoskeleton (Finley et al., 1998).

Similar transport assays have been developed for use in embryos of aquatic invertebrates. Embryos of the echiuran worm *U. caupo* were found to transport rhodamine B, a fluorescent dye and MDR1 substrate, in a verapamil-sensitive manner (Toomey and Epel, 1993). This rhodamine dye efflux assay was used to confirm P-gp-like activity in fertilized oyster (*Crassostrea virginica*) and mussel (*M. edulis*) embryos (Keppler, 1997; McFadzen et al., 1999). Although P-glycoprotein is present in unfertilized *M. edulis* eggs as determined by Western blot, lack of verapamil-sensitive rhodamine transport indicates that there is no P-gp activity until fertilization (McFadzen et al., 1999).

P-gp activity has also been observed in several cell types in fish. P-gp transport activity was investigated in killifish chloride cells within opercular epithelium mounted on Ussing chambers. Rhodamine 123 efflux was inhibited by the P-gp substrate reserpine (Karnaky et al., 1993). Subsequent immunohistochemical analysis has demonstrated P-gp expression in gill chondrocytes in another fish, guppy (Hemmer et al., 1995).

Isolated killifish renal proximal tubules form a closed, fluid-filled luminal compartment that only communicates with the medium through the tubular epithelium, an ideal model for the study of secretion of organic molecules in intact kidney tubules. Epifluorescence microscopy was used to measure the uptake and luminal secretion of the

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fluorescent anthracycline daunomycin by intact killifish renal proximal tubules (Miller, 1995). Luminal fluorescence decreased and cellular accumulation remained unchanged upon exposure to either cyclosporin A or verapamil (Miller, 1995). Transport of a fluorescent cyclosporin analogue in the same system was also blocked by P-gp competitive substrates (cyclosporin A and G, verapamil, vinblastine, quinine) (Schramm et al., 1995). These data provided the first demonstration that P-glycoprotein mediates the secretion of drugs in intact renal proximal tubules. Further functional studies in killifish renal proximal tubules demonstrate that secretion of a large organic anion, fluorescein-methothrexate (FL-MTX), into the lumen was inhibited by cyclosporin A, verapamil and leukotriene C4 (Masereeuw et al., 1996). These compounds may inhibit more that one transport system. Verapamil and cyclosporin A sensitivity indicate P-glycoprotein-mediated transport, while inhibition by leukotriene C4 suggests transport by an MRP family member.

Preliminary studies of cultured rectal gland epithelial cells in dogfish shark (*Squalus acanthias*) suggested P-gp-like activity was responsible for transport of rhodamine dye across the basolateral membrane which was blocked by reserpine, a P-gp inhibitor (Valentich, 1991). The elasmobranch rectal gland is a specialized NaCl and fluid excretory organ composed of columnar epithelial cells arranged as blind-ended branched tubules (Silva et al., 1996). Additional studies in isolated rectal gland tubule fragments found that fluorescent organic anion, sulforhodamine 101 and FL-MTX, were rapidly transported from bath to lumen (Miller et al., 1998a). Efflux was inhibited by the MRP substrate leukotriene C_4 but not by the P-gp inhibitor verapamil. Rectal gland tubules were also found not to secrete P-gp substrates such as daunomycin or fluorescent cyclosporin A derivatives. Immunostaining revealed reactivity of a mammalian MRP2/cMOAT polyclonal antibody to the luminal membrane of epithelial cells. These data suggest that in contrast to studies in cultured cells, intact and isolated shark rectal glands are capable of xenobiotic transport mediated by a shark analog of MRP2, but not by P-gp (Miller et al., 1998a).

6. Physiological function of P-glycoproteins: evidence from gene knockout studies

The generation of knockout mice which lack various P-glycoprotein isoforms has helped elucidate the physiological importance of P-gp expression. The lack of mdr1a in knockout mice has a profound effect on the tissue distribution, elimination, and thus toxicity of P-gp substrate drugs such as vinblastine and ivermectin (Schinkel et al., 1994). In knockouts, vinblastine concentrations were two- to threefold higher in plasma, colon, small intestine, liver, kidney, lung and testis, and 20-fold higher in brain than wild type mice (Schinkel et al., 1994). Exposure to normally benign levels of ivermectin to treat a mite infestation resulted in a 100-fold increased sensitivity. Ivermectin, a neurotoxin, accumulated in the brains of mdr1a (− / −) mice and death quickly followed (Schinkel et al., 1994). These studies suggest P-glycoprotein helps maintain the integrity of the blood–brain and blood–testis barrier in addition to preventing accumulation of xenobiotics in multiple organs.

An outbred mouse stock, CF-1, was discovered to have a 25% incidence of mice phenotypically similar to the mdr1a (− / −) knockouts in that they were deficient in functional P-gp in placenta (Lankas et al., 1998), brain and intestine (Lankas et al., 1997). Pregnant CF-1 mice were exposed during gestation to an isomer of avermectin B1a, a known P-gp substrate and teratogen. Due to the fetal derivation of placental tissue, P-gp genotype of the fetus and not the dam was the significant factor in determining degree of fetal exposure to the P-gp substrate teratogen (Lankas et al., 1997). Homozygous (− / −) fetuses were 100% susceptible to avermectin B1a induced cleft palates, heterozygotes (+ / −) littermates were less sensitive, and homozygous (+ / +) fetuses with normal P-gp levels were resistant to the doses tested (Lankas et al., 1997). These results indicate that placental P-gp creates an important barrier against certain potential teratogens and suggests that P-gp inhibitors could increase susceptibility to chemical-induced teratogenesis.

Double knockout mice mdr1a/1b (− / −) have a 70% decrease in hepatobiliary secretion, and a

90% reduction in intestinal secretion of cationic drugs compared to wildtype (Smit et al., 1998). A compensatory shift to increased renal clearance was observed. The functional role of mdr1b has yet to be elucidated, perhaps mdr1b is more effective than mdr1a in providing protection against as yet unidentified class of xenotoxins (Schinkel, 1997). That biliary elimination of organic cations was not completely abolished indicates that other redundant transport mechanisms may be involved in clearance in addition to type I P-gps.

That mdr1a/1b ($-/-$) are viable and fertile suggests that lack of drug-transporting P-gps is compatible with relatively normal physiology if mice are not exposed to toxins (Schinkel et al., 1994). These studies support the positive expectation that clinical chemosensitizer treatment in cancer patients to block functional P-gp, may not have major effects on human physiology, beyond altered drug metabolism (Borst and Schinkel, 1996).

Mdr2 ($-/-$) knockout mice are unable to secrete phospholipids and cholesterol into the bile and develop liver disease (Smit et al., 1993; Oude Elferink et al., 1996). The liver pathology manifests itself shortly after birth as a nonsuppurative inflammatory cholangitis followed at 4–6 months of age by formation of preneoplastic lesions which progress to terminal metastatic liver cancer (Mauad et al., 1994). Mdr2 knockouts transfected with the human homolog, MDR3, were found to rescue the wildtype phenotype indicating that human MDR3 and murine mdr2 are functionally homologous (Smit et al., 1998). Mdr2 ($-/-$) mice may be valuable models to study both non-suppurative inflammatory cholangitis and progression of hepatocellular carcinomas which are similar in mice and humans.

Recently MRP1 ($-/-$) mice have been generated that suffer from an impaired inflammatory stimulus (Wijnholds et al., 1998) and are susceptible to drug-induced damage to the mucosa of the oropharyngeal cavity, diabetes insipidus, and (temporary) infertility due to inhibition of spermatogenesis (Wijnholds et al., 1997). That all the previously described knockout mice are viable and fertile suggests that either P-gp (and MRP) may not serve a vital function in mice unchal-

lenged by xenotoxins (a situation potentially encountered by lab mice but not wild populations) or that there are other as yet unidentified redundant xenobiotic transport systems. These studies demonstrate the importance of these transporters in maintaining the integrity of the blood–brain, blood–testis, and blood–placental barriers and providing resistance against xenobiotics and toxic endogenous metabolites at many sites throughout the mammalian body.

Multixenobiotic resistance has also been examined in another genetically manipulable organism, the soil nematode *Caenorhabditis elegans*. Four P-gp homologs and four MRP homologs have been identified to date (Lincke et al., 1992; Broeks et al., 1996). Pgp-1, -2, and -3 are expressed in intestinal cells throughout the life cycle of *C. elegans* (Lincke et al., 1993). Nematodes generated with a deleted pgp-1 gene, were sensitive to colchicine and chloroquine (Broeks et al., 1995). Mrp-1 is expressed in the pharynx, intestine, and vulva (Broeks et al., 1996). Nematodes with inactivated mrp-1 are sensitive to heavy metals such as arsenite and cadmium compared to tolerant wild-type worms. Worms with both pgp-1 and mrp-1 deleted were hypersensitive to heavy metals (Broeks et al., 1996). These results suggest that several multixenobiotic resistance proteins may provide nematodes with tolerance to dietary and environmental xenobiotics originating from plants and microbes in the rhizosphere as well as protect them from heavy metals.

7. P-glycoprotein and cytochromes P450

P-glycoprotein, in addition to other xenobiotic transporter, may be a first line of defense against natural toxic products from ingested plants or microorganisms. If the P-gp transporter is overwhelmed by a high dose of a xenobiotic this substrate will accumulate in the cell. Tissues are equipped with a second line of defense: phase I (e.g. hydroxylation via cytochromes P450) and phase II (e.g. conjugation with glutathione) detoxification enzymes which metabolize xenobiotics (as well as endogenous substrates) to more hydrophilic compounds which can be more easily

excreted from the body. An examination of the structure-activity relationships and molecular characteristics for xenobiotic transport substrates and inhibitory ligands of P-gp indicate that P-gp may function in the elimination of hydroxylated metabolites of xenobiotics after modification by phase I enzymes, a phase III elimination process (Bain et al., 1997). Many phase II conjugates are transported by another ABC transporter, MRP. Refer to Fig. 3 for a speculative model for the roles of ABC transporters, phase I and II enzymes in xenobiotic resistance.

P-glycoproteins are expressed in many of the same tissues as the cytochromes P450. Evidence suggests that P-gp transports environmental contaminants or possibly their phase I metabolites including benzo[a]pyrene (Yeh et al., 1992), 7, 12-dimethylbenz(a)anthracene (Phang et al., 1993) and the pesticide endosulfan (Bain and LeBlanc, 1996). Some CYP1A parent compounds and metabolites appear to induce P-glycoproteins in
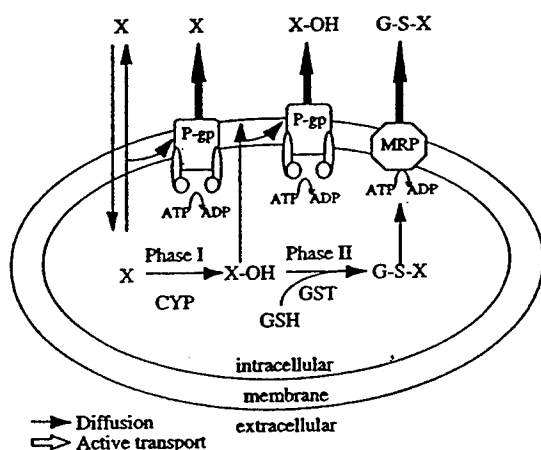

Fig. 3. Speculative model of xenobiotic resistance provided by transmembrane active transporters, P-gp and MRP, and Phase I and II detoxification enzymes, cytochromes P450 (CYP) and glutathione-S-transferase (GST) respectively. A moderately hydrophobic natural product (X) diffuses in and out of the cell and at low concentrations little accumulates due to active efflux of parent compound by P-gp. At high concentrations, X accumulates and is metabolized by one or more of the CYP enzymes. The hydroxylated metabolite is either (i) removed by P-gp mediated transport or (ii) may be further modified by conjugation to glutathione (GSH) catalyzed by GST. The glutathione conjugate (G-S-X) is expelled from the cell by MRP active transport.

mammalian in vitro systems, including benzo[a]pyrene (Yeh et al., 1992), 3-methylcholanthrene (Gant et al., 1991; Fardel et al., 1996), and 2-acetylaminofluorene (2-AAF) (Schrenk et al., 1994). In rats, the P-gp inducers 2-acetylaminofluorene and phenothiazine have been shown to induce some CYP members (CYP1A, CYP2B, and CYP3A2) but not others (CYP2C6, CYP2C11, and CYP2E1) (Tateishi et al., 1999). An early study reported that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) coinduced hepatic P-gp and CYP1A2 in rats (Burt and Thorgeirsson, 1988) but similar exposures in mice did not increase hepatic P-gp suggesting that mdr gene induction does not occur via the aryl hydrocarbon pathway (Gant et al., 1991; Teeter et al., 1991).

These results in mammalian systems encouraged researchers to investigate both P-gp and phase I enzyme expression in aquatic organisms exposed to common environmental contaminants. In one study, CYP1A and P-gp expression were induced in intertidal fish, (Anoplarchus sp.), upon field and laboratory exposures to crude oil (Bard et al., 1998). Crude oil contains components which are substrates and inducers for both CYP1A and P-gp. After a 6 month lab depuration, blennies were exposed to oiled sediment and food for 3 weeks. P-gp expression in the bile canaliculi increased three- to fivefold in oil exposed fish compared to control fish held over clean sediment and fed clean food. P-gp expression was highly correlated to hepatic CYP1A in these fish. Induction of CYP1A and P-gp proteins may be a generalized defense mechanism for intertidal fish commonly exposed to such contaminants (Bard et al., 1998).

In a second study, carp were exposed to water with low concentrations of Diesel-2 oil (Kurelec, 1995b). Induction of CYP1A activity and benzo[a]pyrene monooxygenase (BaPMO) was not detectable in carp liver after 3 day exposures. The addition of the P-gp competitive inhibitor verapamil (2 µM) to the polluted water stimulated large hepatic induction of these mixed-function oxidase activities after 2 days. Verapamil alone did not stimulate induction. Verapamil, by inhibiting P-gp-mediated xenobiotic efflux, in effect

increased the internal dose of Diesel-2 oil in these fish to levels obtained by exposure to fivefold higher concentrations of oil (Kurelec, 1995b).

Additional studies in fish exposed to model CYP1A inducers have produced results that differ from expectations raised by mammalian in vitro studies. *Anoplarchus* sp. exposed to β-naphthoflavone, a model CYP1A inducer, demonstrated no induction of hepatic P-gp (Bard et al., 1998). *F. heteroclitus* exposed to tetrachlorodibenzofuran (up to 50 nmol/kg), displayed strong hepatic induction of CYP1A but none of P-gp (unpublished data from our laboratory). A similar lack of hepatic P-gp induction in the presence of a CYP1A inducer was previously reported in depurated *F. heteroclitus* exposed to 3-methylcholanthrene (3-MC), a model PAH (Cooper et al., 1999). Previously 3-MC was demonstrated to induce P-gp via increased transcription in an in vitro rat primary hepatocyte model but not in vivo possibly due to differences in metabolite formation between the two systems (Gant et al., 1991). Furthermore, in a nonparenchymal rat liver epithelial cell (RLE) model 3-MC exposure induced P-gp but not CYP1A1 nor 1A2 suggesting that these liver detoxification pathways are not coordinately regulated with P-gp in RLE cells (Fardel et al., 1996). Thus patterns of induction appear to depend on experimental system and organism.

Another cytochrome P450 member, CYP3A, may affect P-glycoprotein activity. Currently, information on CYP3A expression in aquatic animals is limited (Celander et al., 1996). Studies in mammalian systems suggest that co-investigation of P-gp and CYP3A in aquatic organisms may be a fruitful area for future work. Many CYP3A substrates or metabolites are also P-gp substrates or inhibitors, including verapamil, vinblastine, vincristine, quinidine, doxorubicin, ketoconazole, digitoxin, progesterone, nifedipine, cyclosporin A, and taxol (Wacher et al., 1995; Zhang et al., 1998). However, a recent in vitro drug screen of CYP3A substrates revealed that some but not all studied compounds were substrates or inhibitors of P-gp (Kim et al., 1999). CYP3A is predominantly expressed in the intestine and liver and is involved in the oxidative metabolism of > 50% of

human drugs (Bertz and Granneman, 1997). Thus P-gp and CYP3A interactions may affect drug and xenobiotic absorption and metabolism (Watkins, 1997). Schuetz et al. demonstrated that lower doses of rifampicin, a P-gp and CYP3A substrate, were required to induce CYP3A in mdr1a (−/−) knockout mice compared to wild-type mice (Schuetz et al., 1996). In colonic carcinoma cells, P-gp and CYP3A4 are both up-regulated by phenobarbital, reserpine, isosafrole, and rifampicin but only P-gp in induced by nifedipine (Schuetz et al., 1996). A survey of human liver bank samples indicated that there was large interindividual variability in the expression of CYP3A4 and MDR1 genes and no apparent correlation in their expression (Sérée et al., 1998). These studies suggest that CYP3A and P-gp may be coinduced in some circumstances.

Several studies have examined the effect of the steroid dexamethosone on CYP3A and P-gp expression in mammals. Dexamethasone treatments of a human hepatoma cell line coinduced both MDR1 and CYP3A4 gene expression (Sérée et al., 1998). Mice exposed to dexamethosone displayed tissue specific responses: mdr1b (fivefold) and CYP3A (twofold) were coinduced in the liver, but adrenal CYP3A increased while mdr1b was repressed (−51%) (Sérée et al., 1998). Disruption of the mdr1 gene in mice does not affect CYP3A protein expression or metabolism of model CYP3A substrates including dexamethasone (Perloff et al., 1999). A recent study reported the first observation of drugs affecting P-glycoprotein expression in a gender specific manner (Salphati and Benet, 1998). In dexamethosone-treated rats, hepatic P-gp was elevated by 50% in males and reduced by 60% in females. These results suggest that CYP3A and P-gp are regulated independently in rat liver and that P-gp expression and regulation is gender specific. Although the mechanisms involved are not elucidated, the gender specific response to dexamethasone might result from effects on a hormonal regulatory pathway.

Although CYPs and P-gp may not be coregulated (Wacher et al., 1998), these studies suggest that they can be coinducible or inversely reduced in a species-, tissue- and gender-dependent manner. CYPs and P-gp may play complementary

roles in xenobiotic and drug disposition by bio-transformation (phase I) and transport (phase III) and act synergistically as a bioavailability barrier (Zhang et al., 1998).

8. Induction and regulation of P-glycoprotein expression

Elevated P-glycoprotein expression may occur via multiple mechanisms including gene amplification, transcriptional and post-transcriptional controls. MDR1 gene amplification has been observed in some resistant cell lines (Roninson et al., 1986) but is not required for increased expression of the gene (Shen et al., 1986). Gene amplification refers to a cell's ability to generate multiple copies of a gene located in extrachromosomal elements (double-minute chromosomes) or within expanded chromosomal regions (homogeneously staining regions or abnormally banded regions). Transcriptional activation may be more relevant in both the clinical setting (van der Heyden et al., 1995) and in wild populations of animals. In the absence of amplification, the increased expression of MDR1 may occur via increased MDR1 mRNA expression (Shen et al., 1986). Post-transcriptional control, such as phosphorylation state and increased mRNA stability/decreased mRNA half-life, is also important for MDR1 expression. MDR1 transcriptional activation may be stimulated by exposure to drugs, xenobiotics, and experimental conditions. Phosphorylation of serines and threonines on P-glycoprotein is associated with elevated drug resistance in mammalian cell lines (Ratnasinghe et al., 1998).

P-glycoproteins induction may be a generalized response to stressful conditions such as xenobiotic exposure or cellular injury. MDR1 mRNA and P-gp may be induced by cellular damage from cytotoxic drugs regardless if the drugs are P-gp substrates or not (Chaundhary and Roninson, 1993). Induction of mdr transcripts in in vitro systems, such as rat liver epithelial cells, can be detected as early as 7 h after exposure to the potent inducer doxorubicin (Fardel et al., 1997). MDR1 induction has been observed to occur in response to exposure to chemotherapeutic drugs

(Chaundhary and Roninson, 1993), DNA-damaging agents (Fardel et al., 1998), retinoic acid (Bates et al., 1989), tumor necrosis factor alpha (TNF-α) (Hirsch-Ernst et al., 1998), protein kinase C agonists (Chaundhary and Roninson, 1993), X-rays (Hill et al., 1990), protein synthesis inhibition (Gant et al., 1992) and contaminants including 2-acetylaminofluorene, carcinogenic polycyclic aromatic hydrocarbons, aflatoxin B1, and arsenite (Fairchild et al., 1987; Chin et al., 1990; Gant et al., 1991; Fardel et al., 1996). Both P-gp and heat shock proteins are controlled by heat shock elements (Chin et al., 1990). Mild heat shock of primary cultured winter flounder renal proximal tubule epithelium led to specific chemical resistance due to induced P-gp-mediated transport of cytotoxic drugs across the apical microvilli (Renfro et al., 1993; Sussman-Turner and Renfro, 1995). Elevation of intracellular reactive oxygen species in primary rat hepatocyte cultures led to induction of mdr1 mRNA and functionally active mdr1-type P-gp while treatment with antioxidants led to repression of intrinsic mdr1b mRNA and P-gp expression (Hirsch-Ernst et al., 1998; Ziemann et al., 1999).

Following partial hepatectomy in rats, expression of P-glycoproteins increased threefold in regenerating livers (Fairchild et al., 1987). Mdr1b induction in rat primary hepatocyte culture was correlated with cellular stress associated with cell isolation and plating (Fardel et al., 1992). Rat hepatic mdr1b expression increased as a function of time in culture due to increased mRNA stability (Lee et al., 1995). Drug-induced biliary cholestasis increased mdr1a and mdr1b transcription in rat liver (Schrenk et al., 1993). In the previously cited examples, when P-gps were identified by isoform, researchers detected induction of class I drug/xenobiotic resistance P-gps which are normally found at low level in the liver compared to the phospholipid flippase mdr2. Mdr2 is normally detected at high levels in bile canaliculi but was not observed to increase in response to treatments in these studies. In contrast, studies in monkeys have demonstrated induction of mdr2 both with and without coinduction of mdr1. Drug-induced biliary cholestasis induced hepatic mdr1 and mdr2 transcription in non-primate

monkeys (Schrenk et al., 1993). Hepatic mdr2 mRNA but not mdr1 mRNA increased in rhesus monkeys in response to xenobiotics or their metabolites that require biliary excretion (Gant et al., 1995). These later studies suggest that mdr2 may play a role in resistance to xenobiotics and/or their phase I metabolites in addition to its phospholipid translocase capacity in these species.

The factors that regulate P-glycoproteins are not fully understood. The following section will outline recent studies which suggest that P-gp expression may be regulated by protein kinase C (PKC) mediated phosphorylation, thyroid hormones, and CFTR protein expression. P-gp transport function can be modulated via phosphorylation catalyzed by PKC. Increased phosphorylation state is associated with increased P-gp function and drug resistance (Center, 1985). Studies of MDR cell lines reveal that PKC activators stimulate P-gp mediated drug efflux, e.g. phorbol 12-myristate 13-acetate (PMA) (Chambers et al., 1990), while PKC inhibitors depress drug transport, e.g. staurosporine (Ma et al., 1991). In vivo studies of aquatic organisms support the suggestion that protein kinases play a role in regulation of P-gp. PKC specific inhibitors, bisindolylmaleimide and staurosporine, inhibits P-gp activity in the marine mussel *M. galloprovincialis* (Kurelec, 1995c). This inhibition was manifested by an increased accumulation of the P-gp substrate ^{3}H-vincristine in mussel gills upon co-exposure to either PKC inhibitor compared to control.

In contrast, an inverse relationship between P-gp-mediated drug transport and PKC activity was reported for studies in teleost fish. The role of PKC in regulation of P-gp in killifish renal proximal tubules was investigated by fluorescence microscopy using isolated tubules (Miller et al., 1998b). Isolated tubules form a closed, fluid-filled luminal compartment that only communicates with the medium through the tubular epithelium, an ideal model for the study of secretion in intact tubules. Furthermore, studies in killifish tubules are a good model for mammalian tubules because both appear to have identical renal secretory transport mechanisms. P-gp-mediated secretion of daunomycin into the tubular lumen was rapidly reduced by low concentrations of PKC activators (PMA and diacylglycerol.) Conversely, protein kinase inhibitors (staurosporine and 1-(5-isquinolinylsulfonyl)-2-methylpiperazine) stimulated secretion. Furthermore, neither PKC activators nor protein kinase inhibitors exerted any effect on steady-state cellular drug accumulation. In addition, PMA decreased transepithelial secretion of [^{3}H]daunomycin in primary cultures of winter flounder proximal tubule cells.

That P-gp mediated xenobiotic secretion is negatively correlated with changes in PKC activity is opposite to previous results in mammalian cell line studies. These data also differ from studies of nonpolar cells which show increased cellular drug accumulation when P-gp is inhibited by chemosensitizers (Ford and Hait, 1990). These results can be interpreted to suggest that cellular uptake of daunorubicin occurred by simple diffusion and that solute efflux into the lumen did not have a major impact on steady-state cellular drug accumulation. The authors of this study propose that the correlation observed between PKC activity and P-gp function in cell lines may not reflect direct phosphorylation of the transporter by PKC but instead an intermediate step between kinase and transporter. Differences in these pathways may account for the differences in PKC effects observed between teleost proximal tubule and mammalian tumor cells (Miller et al., 1998b).

9. Endogenous function of P-glycoprotein

Evidence from recent studies suggests that in addition to toxin evasion, phospholipid and steroid transport, P-gp's endogenous functions may include a role in development and osmotic control (Zucker et al., 1997; Singer, et al., 1999). Studies of P-glycoprotein expression (Xe-mdr) in *Xenopus* provided the first report that a naturally occurring substance can down-regulate mdr gene expression in vivo (Zucker et al., 1997). Studies to investigate Xe-mdr expression during tadpole development revealed that a decline in intestinal Xe-mdr at metamorphosis was correlated with a

natural peak in thyroid hormone secretion. Subsequently, premetamorphic tadpoles which were treated with the hormone triiodothyronine to induce metamorphosis displayed a significant decrease in intestinal Xe-mdr message and protein. Thyroid hormone treatment of primary cultures of *Xenopus* brush border epithelial cells resulted in reduced mdr message in vitro. These studies suggest a role for thyroid hormone in the developmental regulation of intestinal P-glycoprotein in frogs.

Studies suggest that in addition to involvement in osmotic control, MDR1 may be co-ordinately regulated in vivo with the ABC transporter chloride channel CFTR (Trezise et al., 1992). That rodent mdr1 and Cftr show complementary patterns of tissue expression in vivo has been used as evidence that Cftr and P-gp may serve analogous roles in epithelial cells, i.e. to regulate epithelial cell volume (Trezise et al., 1992). Studies of Cftr knockout transgenic mice have demonstrated that a fourfold decrease of intestinal Cftr in homozygous knockouts is accompanied by a fourfold increase in intestinal mdr1 mRNA compared to wildtype mice (Trezise et al., 1997). Heterozygotic mice are phenotypically similar to wildtype but have intermediate levels (twofold increase) of intestinal mdr1. These studies suggests that alterations in mdr1 mRNA are regulated in response to decreased cftr and are not due to a general stress response associated with severe intestinal phenotype of knockout mice (Trezise et al., 1997).

Recently, the first aquatic organism CFTR homolog was isolated from the gill of the killifish, *F. heteroclitus* (Singer et al., 1999). kfCFTR shares 59% amino acid identity with CFTR and is expressed in high levels in the gill, opercular epithelium and intestine. When this euryhaline fish is moved from freshwater to seawater, a sevenfold induction of kfCFTR in gill is observed, which suggests a role in salinity adaptation (Singer et al., 1999). What interactions kfCFTR may have with fPgpA and fPgpB have not yet been studied in the killifish or any other environmentally relevant aquatic organism exposed to variable salinity, an interesting topic for future investigations.

10. Induction of multixenobiotic resistance in aquatic organisms

Numerous studies have reported induction of multixenobiotic resistance transport activity and elevated P-glycoprotein protein levels in (i) field populations of pollutant exposed aquatic organisms, (ii) transplantation experiments, and (iii) laboratory exposures. First, variations in P-gp expression at different field sites have been observed in populations of snails, grass shrimp, mussels, oysters, and fish. Snails, *Monodonta turbinata*, collected from an unpolluted site accumulated 67% more [^{3}H]-vincristine in gill tissue upon inhibition with verapamil than snails from a polluted site, a measure of less overall P-gp mediated efflux (Kurelec, 1995a). Populations of the grass shrimp (*Palaemonetes pugio*) exposed to increased amounts of urban and agricultural runoff in the field were shown to express higher levels of P-glycoprotein (Finley et al., 1996; Scott et al., 1999) *Mytilus galloprovincialis* from polluted sites accumulate 46% less verapamil than unpolluted specimens (Kurelec, 1995c). The level of MXR protein expression between populations of oysters (*C. gigas*) and mussels (*M. edulis*), measured via protein dot blots varies with levels of organic pollutants at sample sites (Minier et al., 1993).

P-gp in oyster gills (*C. virginica*) was elevated at organically polluted sites compared to reference sites (Keppler and Ringwood, 1996). Interestingly, at all sites, levels of P-pg expression were seasonal with elevations in summer and fall (Keppler, 1997). P-gp seasonal expression was not correlated with exposure to any specific organic sediment pollutant but was coincident with elevated seawater temperature and algal blooms. Heat shock or elevated natural products in the water column from algal blooms may be responsible for seasonal P-gp expression observed (Keppler, 1997).

Immunoblot and immunohistochemical studies of P-gp expression have been conducted in nonmigratory fish species which have a small home spot and thus reflect xenobiotic exposure at the site where they were collected. A population of resistant *F. heteroclitus* exposed to PAHs in the field at a creosote contaminated site are reported

to have a two to threefold induction of hepatic P-gp compared to fish from a reference site (Cooper et al., 1999). Large individual variability has been observed in hepatic P-glycoprotein levels in freshly-caught fish (*Anoplarchus purpurescens* and *F. heteroclitus*) such that the standard deviation is equal to the mean (Bard et al., 1998; Bard and Stegeman, 1999). These results suggest that individual fish may have variable abilities to respond to P-gp inducers.

Field and laboratory exposures to xenobiotics induce higher levels of MXR activity and P-gp protein in several aquatic organisms. Two methods can be used to measure P-gp transport activity: bioaccumulation assays and efflux assays. In the first case, the accumulation of a fluorescent substrate, e.g. rhodamine dye, is measured with or without the presence of a competitive inhibitor in translucent living cells (e.g. mussel gill or blood cells or worm embryos) by fluorescent microscopy (Toomey and Epel, 1993; Cornwall et al., 1995; Minier and Moore, 1996a,b) or in tissue homogenates prepared after the exposures were completed by a fluorometric plate reader (Smital and Kurelec, 1998a,b). In efflux assays, the rate of efflux of a previously accumulated fluorescent substrate into the medium is measured in the presence or absence of a competitive inhibitor (Smital and Kurelec, 1998a,b). The efflux rate is directly proportional to P-gp transport activity. This assay is simple, inexpensive, and animals are not injured by the procedure thus allowing individuals to be used repeatedly in different experiments.

Both methods were found to give similar results in a study of P-gp activity inducibility in mussels (*M. galloprovincialis*) exposed to seawater contaminated with Diesel-2 oil, rhodamine 123, or to polluted field sites (Smital and Kurelec, 1998a,b). P-pg activity in gills increased linearly with exposure to oil or dye, reaching a maximum at day 4 in which efflux rate were 45–50% lower than day 0. In mussels transplanted from a clean to polluted sites, gill P-gp transport activity increased 43% by day 4, equaling the levels found in native mussels at this site. And mussels transplanted from the polluted to clean site showed a decrease in P-gp activity to the level of native pristine mussels also by day 4.

To determine the inducibility of P-gps by environmental xenobiotics, marine mussels (*M. californianus*) were exposed to acetylaminofluorene, an MXR substrate for 24 h. A slight though statistically significant induction of P-glycoprotein activity was detected by the rhodamine dye efflux assay (Eufemia and Epel, 1996). Mussels were also exposed to subacute levels of water-borne toxins for 3 days (Eufemia and Epel, 1998). Both P-gp substrates (e.g. chlorthal pesticide) and non-substrates (e.g. Arochlor 1254) were able to increase gill P-gp protein titer and P-gp-mediated efflux of rhodamine dye from mussel gill cells (Eufemia and Epel, 1998).

Mussels (*M. edulis*) which were exposed to a known P-gp inducer, vincristine (Meyers et al., 1985), by adductor muscle injections demonstrated increased P-gp expression in both gill and blood cells (Minier and Moore, 1996a,b). An assay was developed to measure P-gp transport activity via the capacity of mussel blood cells to efflux a P-gp substrate, e.g. rhodamine B or calcein AM (Minier and Moore, 1996a,b, 1998). Blood cells from treated animals were able to lower their intracellular concentration of the P-gp substrate rhodamine B in a dose dependent manner. P-gp induction which was stable for 14 days. A valuable aspect of this assay, is the ability to use the same individual animals repeatedly. This same assay was used to assess P-gp activity in mussel blood cells from two field sites. Mussels from a PAH contaminated site expressed more P-gp protein and transport activity (29% less rhodamine B and 25% less calcein accumulation) in blood cells than a reference site where mussels accumulate 10-fold less PAH in their tissues (Minier and Moore, 1996a,b, 1998).

MXR induction upon xenobiotic exposure has also been observed in channel catfish, snails, and clams. Transport of [³H]-vinblastine in membrane vesicles prepared from channel catfish intestinal tissue is verapamil-sensitive, suggesting P-gp-like activity (Doi et al., 1999). In vivo induction of a C219 reactive protein is detected in the luminal mucosa of the distal intestine of channel catfish after 10 day dietary exposure to benzo[a]pyrene or TCB (3,4,3′,4′-tetrachlorobiphenyl) (Doi et al., 1999.)

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The marine snail *Monodonta turbinata*, upon transplantation to a polluted site for 3 days or exposure to Diesel-2 oil for the same period of time, accumulated less vincristine (14.5 and 55%, respectively) and lost sensitivity to the inhibitor verapamil thus indicating an induction of MXR and increased translocation of P-gp substrates (Kurelec, 1995a). In addition to other detoxification systems (e.g. phase I–III enzymes), the induction of a multixenobiotic defense mechanism in organisms living in polluted environments may explain why contaminant spills cause more severe adverse effects in pristine sites than in already polluted areas (Kurelec, 1995a).

MXR activity in freshwater clams (*Corbicula fluminea*) was measured via capacity to decrease accumulation of vincristine (VCR) from clam gill cells (Kurelec et al., 1996). Control clams that were exposed to Rhine River sediments or Diesel-2 oiled water for 3 days accumulated significantly less VCR than unexposed clams. Clams freshly collected from a polluted Rhine River site accumulated 41% less VCR than depurated control clams held in clean aquaria for 6 weeks. Surprisingly, Western blot analysis detected no difference in P-gp protein levels suggesting that modulation of MXR activity in clam may either be caused by some modification of the existing protein (Kurelec et al., 1996) or that another xenobiotic transport system may be responsible for the observed efflux of vincristine.

11. Chemosensitizers as environmental pollutants

Both natural products and anthropogenic contaminants appear to be substrates for the P-glycoproteins present in aquatic organisms. The previously describe rhodamine dye efflux assay has been modified to evaluate potential MXR substrates in vivo using worm embryos, mussel and clam gills. P-glycoprotein substrates were characterized by their ability to competitively inhibit the efflux of rhodamine leading to a measurable accumulation of dye within the cells of the model organisms.

Unpurified hydrophobic metabolites of bacteria isolated from the gut of a marine worm (*U.*

caupo) and from the sediment in its burrow (Toomey and Epel, 1993; Toomey et al., 1996) were able to inhibit rhodamine dye efflux from fertilized worm embryos. Enhanced accumulation of dye within embryos was also observed after exposure to moderately hydrophobic pollutants (sulfallate (CDEC), dacthal (DCPA), pentachlorophenol, 2-acetylaminofluorene) (Toomey et al., 1996). As expected, more hydrophobic contaminants [dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethane (DDD), dichlorodiphenylchloroethane (DDE), polychlorinated biphenyls, benzo[a]pyrene] did not affect dye transport.

This dye assay was further modified for the gill tissue of *M. californianus* and *M. galloprovincialis* to evaluate a variety of environmental compounds monitored in the Mussel watch program as potential MXR substrates or inhibitors (Cornwall et al., 1995; Galgani et al., 1995). Several moderately hydrophobic pesticides including chlorbenside, sulfallate, dacthal, and pentachlorophenol inhibit dye efflux in mussel gills cells indicating that these compounds are either substrates or inhibitors (Cornwall et al., 1995; Galgani et al., 1995). These compounds were furthermore observed to interact with MXR at nanomolar concentrations similar to what mussels would be exposed to in the aquatic environment. More hydrophobic xenobiotics such as DDT, DDD, DDE, and Aroclor 1254 do not inhibit dye efflux in the mussel assay as expected, suggesting they do not interact with P-gp.

Using the assay, algal products were assessed to be naturally occurring modulators for the multixenobiotic resistance transporter in mussels (*M. californianus*) (Eufemia et al., 1999). Rhodamine B accumulates within gill cells when in the presence of methanol extracts of macroalgae (*Macrocystis pyrifera*, *Egregia menziesii*, *Phyllospadix scouleri*, *Gracilaria sp.*, *Gigartina sp.*) or ethanol extracts of phytoplankton which produce the neurotoxins saxitoxin or domoic acid (*Alexandrium catanella* and *Pseudonitzchia australis*, respectively). These extracts also modulated P-glycoprotein transport activity in MDR human tumor cell lines. These data suggest that filter feeding mussels may have evolved high levels of gill P-gp

in response to the large load of MXR substrates in a diet dominated by seaweed particulates and phytoplankton (Eufemia et al., 1999).

The MXR inhibitory potential of seawater and river water was assessed by measuring rhodamine accumulation and efflux from treated gills of marine mussels (*M. galloprovincialis*) and freshwater clams (*Dresissena polymorpha*) (Smital and Kurelec, 1997). In the efflux assay, bivalves are exposed to rhodamine B and putative substrates (water samples) and the medium is sampled over time to measure dye eliminated from the organism. The efflux assay is directly proportional to P-gp activity and does not require that the organism be killed thus permitting the same individual to be used repeatedly. Higher concentrations of MXR inhibitors were detected in samples of river and seawater from polluted sites than from unpolluted sites. Both methods gave similar results, polluted waters enhanced accumulation or decreased efflux rate of dye compared to clean waters. Unidentified components of the polluted seawater (Smital and Kurelec, 1997) were evaluated to be potential P-gp substrates using a similar dye assay on an MDR cell line. Natural water was found to inhibit MXR as effectively as extracts of the moderately hydrophobic compounds in the water samples, leading to the conclusion that the easier method (simply using water samples straight without further processing) was preferable. The efflux assay was recommended as the simplest, least expensive, and most reliable method for measuring MXR in appropriate test organisms in vivo.

In addition to toxic environmental contaminants and natural product aquatic toxins, innocuous moderately lipophilic substances found in household waste or municipal wastewater may be MXR modulators (Kurelec et al., 1998). Dichloromethane-methanol extracts were prepared from solid waste sampled from a municipal landfill in Zagreb, Croatia which stores household and industrial waste separately. MXR modulation was measured as the potential for extracted waste to increase cellular accumulation of calcein AM in cultured MDR1 transfected mouse fibroblasts. Greater MXR inhibition was observed in samples collected from a segment of landfill dedicated to household waste compared to samples taken from the portion of the landfill reserved for industrial waste. The highest levels of chemosensitizers were detected in fermented household wastes. Chemosensitizing potential was correlated to dissolved organic carbon (DOC) content but correlated with neither the extract's mutagenicity, nor levels of 48 identified PAHs, nor its potential to induce hepatic benzo[a]pyrene monooxygenase in exposed carp.

In a related study, MXR modulation was measured as the ability of a water sample to decrease the in vivo efflux of rhodamine B from freshwater mussels as compared to verapamil (*Dreissena polymorpha*) (Kurelec et al., 1998). Riverwater sampled upstream of a site polluted with untreated municipal wastewater inhibited dye efflux 26% more than water sampled from an unpolluted river. Riverwater sampled 500 m downstream from the effluent discharge had a MXR inhibitory potential 278% greater than the unpolluted riverwater. Modulatory strength was related to DOC content, with sample from discharge site (76 mg/l) far exceeding levels measured upstream (2.4 mg/l) or from the unpolluted river (0.8 mg/l). These results demonstrate that organic molecules in household waste that are not categorized as traditional pollutants (e.g. mutagens or inducers of liver detoxification enzymes) may disrupt multixenobiotic resistance (Kurelec et al., 1998).

Hexane extracts of water from several river sites with variable levels of pollution have been screened for MXR chemosensitizer activity by binding and efflux assays using mammalian model systems (Kurelec et al., 1995a). Water from three rivers was sampled and the state of pollution was defined by levels of benzo[a]pyrene monooxygenase activity in local fish: Korana River (3.4 pmoles) < Sava (22 pmoles) < Rhein (26 pmoles.) The binding assay measures the capacity of membrane vesicles from P-gp-rich bovine adrenal glands to bind [G-^{3}H]vincristine in the presence of water extracts as compared to the model substrate verapamil. The efflux assay measures efflux of the labeled substrate from cultured MDR cells. The two assays noted similar trends for levels of chemosensitizers which correlated with original estimates of pollutant loads. The more accurate

efflux assay measured chemosensitizer loads lowest at Korana < < Sava (+150%) < Rhein (+177%.) This high level of chemosensitizers in the most polluted river sampled may be able to revert the natural resistance of aquatic organisms in this river to a pathobiological sensitivity (Kurelec et al., 1995a). That many of these compounds inhibit MXR activity in vivo in aquatic organisms and in vitro in mammalian vesicle preparations and MDR cell lines suggests that some of the less toxic aquatic natural products may be candidate clinical chemosensitizers.

12. Ecotoxicological consequences of P-glycoprotein inhibition

Exposure to either competitive inhibitors (e.g. verapamil or vincristine) or indirect chemosensitizers (e.g. PKC inhibitor staurosporine) can enhance the accumulation of xenobiotics that are normally transported by P-gp. Studies in aquatic organisms have demonstrated that both known chemosensitizers and environmental pollutants can lead to increased accumulation of P-gp substrates. Clams (*C. fluminea*) exposed to staurosporine accumulate sevenfold more VCR than uninhibited clams (Waldmann et al., 1995). Sponge cubes (*Tethya aurantium*) exposed to radiolabeled environmental carcinogen benzo[a]pyrene contaminated seawater accumulated 73% more label upon exposure to verapamil (Kurelec and Pivcevic, 1992). Mussels (*M. galloprovincialis*) exposed to Diesel-2 oil accumulate threefold greater [^{3}H]-vincristine (VCR) than upon exposure to VCR alone (Kurelec, 1995c). Sediment pore water and riverwater concentrates collected for both a polluted and an unpolluted site all highly inhibited VCR efflux in mussels (Kurelec, 1995c). These results suggest that in addition to organic pollutants, unidentified environmental xenobiotics can reverse multixenobiotic resistance.

The inhibition of P-gp can result in an elevated internal dose of xenobiotics which can alter detoxification enzyme activities. Inhibition of P-gp by verapamil (0.5 μM) in carp exposed to water polluted with Diesel-2 oil enhanced the internal dose of Diesel-2 oil. Liver CYP1A and BaPMO activity were induced to levels otherwise reached after exposure to five times higher concentrations of oil (Kurelec, 1995b).

In the case of genotoxins, P-gp inhibition has been demonstrated to enhance mutagenic and carcinogenic effects in three studies of sponges, clams, and mussels. First, AAF-DNA adducts are elevated in sponge (*G. cydonium*) cubes exposed to seawater treated with AAF and verapamil (270 cpm) than in sponge exposed to AAF alone (160 cpm) (Kurelec, 1992). Second, clams (*C. fluminea*) freshly collected from a polluted Rhine river site demonstrated fewer DNA single strand breaks (SSB) in their gills upon AAF exposure (0.01 μM) than depurated clams held in clean aquaria for 6 weeks (Kurelec et al., 1996). When clams were exposed to oil experimentally, 43% fewer SSB were detected in induced clams freshly collected from the polluted site than depurated control clams (Kurelec et al., 1996). If freshly caught clams are co-exposed to staurosporine and 0.01 μM of AAF, SSB are detected at levels equivalent to that measured in clams exposed to 10-fold more AAF (0.10 μM) (Waldmann et al., 1995). Thus inhibition of P-gp, increases internal dosing of a genotoxin by an order of magnitude in clams. Finally, freshwater mussels (*Dreissena polymorpha*) were exposed to water spiked with 2-aminofluorene (AF.) Co-incubation with cyclosporin (10 μM) enhanced production of ultimate mutagens in exposure medium by 460% (Kurelec et al., 1998).

Freshwater fish (*Leuciscus idus melanotus*) which were exposed to Rhine River water spiked with 2-aminoanthracene (AA) exhibited liver DDN adducts compared to no detectable SSB in fish exposed only to river water. Co-exposure to verapamil and AA, increased the formation of AA-related hepatic DNA adducts in fish. Exposure to river water spiked with non-carcinogenic levels of verapamil (1.5 μg/ml) yielded an increase in SSB.

In this experiment, inhibition of P-gp not only resulted in DNA alterations but also altered animal behaviour. Exposure to behaviourally nontoxic levels of verapamil (1 μg/ml) in river water stimulated fish to make dramatic escapes attempts

which escalated to suicidal escaping behaviour when AA was added to the medium (Kurelec, 1992). This observation is the first report of modified animal behaviour associated with inhibition of P-glycoprotein.

Exposure to non-toxic concentration of P-gp substrate drugs in the presence of chemosensitizers may result in cytotoxic consequences for organisms during for example, the sensitive embryonic stage. Worm embryos (*U. caupo*) were incubated with emitine or vinblastine at concentrations that do not effect the rate of cell division (Toomey and Epel, 1993). Upon co-incubation with the competitive inhibitor verapamil, embryos exposed to 2 µM emitine showed a two- to threefold decrease, while a 4 µM exposure suppressed cell division by fourfold. Even more dramatic effects were observed in embryos co-exposed to vinblastine and verapamil: 0.2 µM depressed cell division four to fivefold, while 0.5 µM vinblastine caused a 350-fold decrease (Toomey and Epel, 1993.) Similarly, mussel embryos (*M. edulis*) which were exposed to vinblastine, mitomycin-C, cytocholasin D, chloroquine, or colchicine showed an increase in number and severity of deformities when P-gp was competitively inhibited by addition of verapamil (McFadzen et al., 1999).

Normally benign levels of an environmental pollutant may accumulate to cytotoxic levels in organisms exposed to natural product chemosensitizers. The invasive green alga *Caulerpa taxifolia* was accidentally introduced to the Mediterranean Sea in 1984 and since that time has detrimentally affected the richness and diversity of littoral algal communities (Verlaque and Fritayre, 1994). The addition of hydrophobic extracts from *C. taxifolia* to an MDR cell line was found to increase accumulation of calcein AM (3.4-fold) and rhodamine 123 (1.5-fold) more than an equal concentration of verapamil (Smital et al., 1996). Furthermore the extracts inhibited P-gp-ATPase activity 2.4-fold stronger than an equal concentration of cyclosporin A (Smital et al., 1996). Extracts of *C. taxifolia* or the purified toxin caulerpin was found to inhibit the P-gp mediated efflux of rhodamine B from an MDR cell line and mussel gills (*Dreissena polymorpha*) (Smital et al., 1996; Schröder et al., 1998). The common water pollutant trib-

utyltin induces apoptosis in the marine sponge (*Geodia cydonium*) at ≥ 3 µM concentrations. Sponges exposed to non-toxic concentrations of *C. taxifolia* or caulerpin with a normally non-toxic dose of tributyltin (1 µM) undergo pronounced apoptosis. The synergistic enhanced toxicity of this aquatic pollutant may be due to inhibition of P-gp by the algal toxin (Schröder et al., 1998). These algal products may be candidate clinical chemosensitizers for in vivo reversal of MDR in cancer chemotherapy.

13. Conclusions

P-glycoprotein expression may be a fundamental mechanism to protect organisms from intracellular accumulation of a wide variety of moderately hydrophobic endogenous metabolites, natural and anthropogenic toxins. The induction of P-glycoproteins, in addition to phase I–III detoxification mechanisms, in organisms living in polluted environments may explain why contaminant spills cause more severe adverse effects at pristine sites than in already polluted areas. The results of the presented studies necessitate a reevaluation of aquatic monitoring programs, such as the Mussel Watch, which measures the presence of contaminants in hardy bivalves. Elevated P-gp expression in mussels permits these organisms to protect themselves from monitored xenobiotics (Cornwall et al., 1995). Thus, absence of a P-gp substrate in a multixenobiotic resistant organism does not signify absence of the compound in the environment. Current monitoring programs may underestimate the environmental concentration of chemicals that are P-gp substrates (Cornwall et al., 1995). Although these chemicals may not accumulate to high levels in multixenobiotic resistant organisms, other sensitive organisms, for example, sea urchins may not express this protective mechanism to the same extent as resistant species (Toomey and Epel, 1993).

Anthropogenic environmental contaminants and natural product which inhibit P-glycoproteins make up a class of pollutants dubbed *chemosensitizers* (Kurelec, 1995b). Benign xenobiotics with a

high affinity for P-gp may inhibit efflux of low levels of toxic compounds which normally would not adversely affect the cell. Toxins normally removed by P-gp now accumulate, elevating the internal dose, and may exert cytotoxic, genotoxic or neurotoxic effects at environmental levels not regularly considered harmful. Effluent and environmental contaminants should be screened for chemosensitizing potential and the levels of these compounds monitored in the environment to avoid inhibiting P-gp in aquatic organisms. Exposure to anthropogenic waste may make aquatic organisms susceptible not only to anthropogenic pollutants but also to natural product toxins. These compounds are products of the chemical warfare between plants and animals which may have stimulated the evolution of resistance mechanisms, such as P-gps. For example, exposure to chemosensitizers might increase a fish's sensitivity to an algal toxin such as okadaic acid, a known P-gp substrate. In addition, expose to chemosensitizers may inhibit the P-gp-mediated elimination of endogenous substrates resulting in pathology analogous to, for example, liver disease observed in mdr2 (− / −) knockout mice (Smit et al., 1993; Mauad et al., 1994.)

Furthermore, some researchers have suggested that the energy cost to sustain a perpetual state of toxin resistance may ultimately reduce overall fitness of organisms in a chemically stressful environment with a heavy P-gp substrate/chemosensitizer load (Renfro et al., 1993; Epel, 1998). A better understanding of multixenobiotic resistance in the aquatic ecosystem will also be useful for fisheries and aquaculture managers. A food animal's ability to accumulate or excrete substances toxic to humans would certainly alter the food value of the organism. The environmental management of emissions of chemosensitizers would benefit both wild aquatic species and cultivated species for human consumption.

Much work remains to be done to characterize the function of P-gps in wild populations of aquatic organisms, and to determine how P-gps interact with other detoxification systems (e.g. phase I and II enzymes) and xenobiotic transporter (e.g. MRPs.) Studies of multixenobiotic resistance in aquatic organisms may have implica-

tions for human health. Wild populations of fish documented to be resistant to anthropogenic contaminants or natural product toxins, may prove useful models for studying what role P-gps play in providing multixenobiotic resistance. The toxin exposure history of coastal fish more closely resembles that of humans than rodents maintained in laboratories and fed pristine food. Elucidating the induction and regulation of P-gp in field animals and discovery of non-toxic natural product chemosensitizers from the aquatic environment may provide important information for cancer chemotherapeutic strategies and new drug development.

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Chapter 3

P-glycoprotein and cytochrome P450 1A expression in winter flounder (*Pleuronectes americanus*) cholangiocellular carcinomas

Abstract

P-glycoproteins (P-gps) are well known to confer multidrug resistance in tumor cell lines and some human tumors, although rare tumor types have seldom been examined. We examined P-gp expression in liver of winter flounder (*Pleuronectes americanus*) from a highly contaminated site in Boston Harbor, Massachusetts. This population was previously shown to have a high prevalence of cholangiocellular carcinomas associated with foci of hydropically vacuolated cells derived from biliary preductular cells. In contrast to expectations based on the classical multidrug resistance phenotype of elevated P-gp levels being commonly observed within hepatocellular carcinomas in mammals and fish, P-gp was highly expressed in bile canaliculi of non-tumorous liver parenchyma surrounding the cholangiocellular carcinoma, but was only detected within the carcinoma of one individual. None of the non-tumor bearing fish from Boston Harbor exhibited detectable levels of canalicular P-gp and only one fish from Cape Cod Bay, where tumors have not been found, stained for P-gp. No P-gp staining was detected in hydropically vacuolated cells, present in tumor bearing liver. We also investigated whether levels of P-gp expression might be related to cytochrome P450 1A (CYP1A) induction in these fish. CYP1A expression, which is induced by contaminants in Boston Harbor and Cape Cod Bay, was detected in the hepatocytes and endothelial cells in normal liver tissue in 100% of Cape Cod Bay flounder, 100% of non-tumorous Boston Harbor fish, and 87% of tumor

bearing Boston Harbor fish. No CYP1A staining was detected in hydropically vacuolated cells. The same fish which expressed P-gp within its cholangiocellular carcinomas was the only specimen to have detectable CYP1A expression in tumor tissue. The lack of correlation between CYP1A and P-gp expression in the winter flounder populations we examined suggests that exposure to environmental contaminants in Boston Harbor, including CYP1A inducers, did not stimulate elevated P-gp in normal liver parenchyma of tumor bearing fish. Rather, it would appear that the presence of hepatic disease in these fish may be responsible for increased P-gp expression in hepatic parenchyma. We suggest that cellular stress caused by impaired bile elimination, possibly linked to the presence of the cholangiocellular carcinomas in these fish, may be responsible for the elevated P-gp observed in the normal liver parenchyma surrounding tumors.

Keywords: P-glycoprotein, cytochrome P450 1A, cholangiocellular carcinoma, multidrug resistance, multixenobiotic resistance, fish

Introduction

P-glycoproteins (P-gps) are well known to confer multidrug resistance in tumor cell lines (Juliano and Ling, 1976) and some human tumors (Gerlach et al., 1986), although rare tumor types have seldom been examined. P-glycoproteins function as energy dependent efflux flippases that prevent the cellular accumulation of a wide variety of moderately hydrophobic compounds. Known P-gp substrates include endogenous regulators such as glucocorticoids (Naito et al., 1989, Ueda et al., 1992), drugs and other natural products (Gottesman and Pastan, 1988), and environmental contaminants (Phang et al., 1993, Cornwall et al., 1995, Bain and LeBlanc, 1996). Exposure to environmental contaminants which are known carcinogens is associated with development of hepatic neoplasms in several bottom-feeding fish species (reviewed by Moore and Myers, 1994).

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In some fish populations, the most common hepatic neoplasms are seen uncommonly in humans, offering an opportunity to evaluate P-gp expression in these tumor types, and possibly to assess environmental influences on P-gp expression.

Winter flounder (*Pleuronectes americanus*) is among those species in which hepatic tumors have been identified in natural populations. In particular, a population of this species in Boston Harbor exhibited liver tumors associated with exposures to contaminants (Moore and Stegeman, 1994); both the sediments (Boehm, 1984, Shiaris and Jambard-Sweet, 1986, NOAA, 1988) and winter flounder (Moore *et al.*, 1996) from Boston Harbor previously contained high levels of polycyclic aromatic hydrocarbons (PAHs), halogenated aromatic hydrocarbons (HAHs), and heavy metals, many of which are suspected carcinogens. The tumors in liver of winter flounder were predominantly cholangiocellular carcinomas, evident in 10% of flounders collected over several years (Murchelano and Wolke, 1985, Murchelano and Wolke, 1991, Gardner and Pruell, 1988, Moore *et al.*, 1997). These hepatic tumors were associated with foci of hydropically vacuolated cells (Bodammer and Murchelano, 1990) thought to be derived from biliary preductular cells (Moore *et al.*, 1997). These vacuolated cells are spatially and may be mechanistically linked to hepatocarcinogenesis in these fish.

The goal of this study was to describe the expression of P-gp in advanced cholangiocellular carcinomas and vacuolated cells of winter flounder compared to expression in surrounding non-tumorous tissue and to livers of non-diseased fish. P-glycoprotein-like genes have been described in winter flounder (Chan *et al.*, 1992). We examined archived liver samples from both tumor and non-tumor bearing winter flounder collected in 1988 from Deer Island Flats, Boston Harbor, Massachusetts. We also examined specimens of winter flounder from the relatively uncontaminated site of Eastern Cape Cod Bay; fish from this site rarely exhibit liver hydropic vacuolation or hepatic neoplasms (Moore *et al.*, 1997). We also investigated whether levels of P-gp expression

might be related to cytochrome P450 1A (CYP1A) induction in these fish. The phase I detoxification enzyme CYP1A is induced by PAHs and planar HAHs, and also catalyzes the oxidative metabolism of these contaminants which renders them more polar and hence more readily eliminated from the body, possibly by efflux transporters such as P-gps. Whether P-gps play a more important role in the transport of CYP1A metabolites rather than parent compounds has not been entirely elucidated (Phang *et al.*, 1993, Bain and LeBlanc, 1996). It is also unclear whether CYP1A inducers/substrates might induce P-gp.

Our results indicate that P-gp and CYP1A expression is low in cholangiocellular carcinomas and vacuolated cells of winter flounders from the contaminated site. Both tumor and non-tumor bearing fish from both sites have elevated CYP1A levels in normal hepatocytes and hepatic endothelium. Unexpectedly, P-glycoprotein expression is elevated in the normal liver parenchyma surrounding the cholangiocellular carcinomas. We suggest that cellular stress caused by impaired bile elimination possibly linked to the presence of advanced cholangiocellular carcinomas in these fish may be responsible for elevated P-gp observed in the normal liver parenchyma surrounding tumors.

Materials and Methods

Archived winter flounder samples

For our study, we examined archived samples which had been previously obtained (Moore *et al.*, 1996). In the original study, adult winter flounder were collected by otter trawl from two field sites in Massachusetts: Deer Island Flats in Boston Harbor the nearest flounder habitat to the Deer Island sewage outfall (1988), and a reference site in eastern Cape Cod Bay between Wellfleet and Provincetown (1996). Upon capture, fish were sacrificed by cervical scission, livers were removed and visually examined for the presence of tumors. For fish without visible tumors, a portion of liver was removed and immediately fixed in neutral buffered formalin. For tumor-bearing fish, a portion of liver

was cut to include both the tumor and surrounding parenchyma followed by tissue fixation. Liver specimens were embedded in paraffin upon return to the laboratory. Livers from 21 flounder from Boston Harbor were examined: livers of 14 fish which contained both hepatic hydropic vacuolation and advanced cholangiocellular carcinomas; and livers of 7 fish that had neither condition. The livers of 10 flounder collected from Cape Cod Bay contained neither hydropic vacuolation nor tumors. Samples were defined as the cholangiocellular carcinoma and surrounding parenchyma or the liver from a non-tumor bearing fish. The progressive stages of hydropic vacuolation and the pathology of the cholangiocellular carcinomas in these specimens have been previously described (Moore and Stegeman, 1994, Moore *et al.*, 1996).

Immunohistochemical analysis
Cytochrome P450 1A detection

Paraffin-embedded samples were sectioned at 5μm on Super-frost plus slides, deparaffinated, hydrated, and immunochemically stained using an indirect peroxidase stain (Universal Immunoperoxidase Staining Kit (Murine), Signet Laboratories, Inc., Dedham, MA) with monoclonal antibody (mAb) 1-12-3 to scup cytochrome P4501A as the primary antibody, as described previously (Smolowitz *et al.*, 1991). Previous immunofluorescence studies have demonstrated the specificity of mAb 1-12-3 for P4501A in tissue sections by immunoadsorption (Miller *et al.*, 1988).

P-glycoprotein detection

Procedures were similar to cytochrome P450 1A detection except for the following changes which were adapted from Miller *et al.* (Hemmer *et al.*, 1995). Sections were mounted on poly-L-lysine-coated slides (Sigma, St. Louis, MO), deparaffined, and then hydrated with 0.5% bovine serum albumin/0.5% nonfat dry milk in TPBS (0.05% Tween

20 in PBS). The affinity purified murine mAb C219 (IgG2a) (Signet Laboratories, Inc., Dedham, MA) which is immunoreactive against the mammalian P-gp (Kartner *et al.*, 1985) served as the primary antibody (10 μg/ml in TPBS). C219 recognizes an internal, highly conserved amino acid sequence common to human MDR1 and MDR2(3) of the 170 kDa transmembrane glycoprotein, as well as the sister of P-glycoprotein (spgp) also known as the bile salt export pump (BSEP), and all other mammalian and non-mammalian mdr isoforms whose sequence is known (Kartner *et al.*, 1985, Endicott and Ling, 1989, Georges *et al.*, 1990), including two winter flounder P-glycoproteins (Chan *et al.*, 1992). Monoclonal antibody C219 has been used previously to recognize P-glycoprotein expression in winter flounder renal proximal tubules (Sussman-Turner and Renfro, 1995).

Stain quantification

Specific staining by mAb 1-12-3 or mAb C219 was evaluated by light microscopic examination of the stained sections. Cell types that stained and their associated occurrence and staining intensity were recorded for each tissue section examined. Staining results were recorded as intensity in any one cell type compared to reference specimens previously designated to stain with either high or low intensity: negative (0), mild (1), mild/moderate (2), moderate (3), strong (4) or very strong (5); and as the degree of occurrence in any one cell type: absent (0), rare (1), some (1.5), many (2) and all (3). Quantitative comparisons were made between tissue types at various sites by using the product of intensity and occurrence, giving a range of score from 0 to 15 staining index. This scoring scheme has been described previously (Woodin *et al.*, 1997).

Statistical Analyses

Differences between reference and field sites and between control and treatment group means were statistically analyzed by one-way ANOVA using Dunnett's one-tailed

procedure for unequal sample sizes using the SuperANOVA (Abacus Concepts) statistical

program; p≤ 0.05 was accepted as significant.

Results

Immunohistochemical detection of P-glycoprotein

P-glycoprotein staining by mAb C219 was localized to the bile canaliculi in normal liver and in the non-tumorous hepatic parenchyma surrounding cholangiocellular carcinomas (Figure 2). Eighty-seven percent of the tumor bearing livers of fish from Boston Harbor (12/14) expressed P-gp at relatively high levels (5.5 ± 4.6 staining index) in bile canaliculi in the parenchyma surrounding the carcinoma (Figure 3). In comparison, none of the livers of fish from Boston Harbor which did not contain tumors had exhibited detectable levels of P-gp and only one fish from Cape Cod Bay stained for P-gp (Figure 3). No P-gp staining was detected in the hydropically vacuolated cells in any sample (Figure 3). Elevated P-gp levels commonly are observed within hepatocellular carcinomas in mammals (reviewed by Bradley and Ling, 1994) and fish (Köhler *et al.*, 1998, Cooper *et al.*, 1999). In contrast to that expectation, P-gp was detected within cholangiocellular carcinoma cells in only one fish, and that expression was at a low level. A second cholangiocellular carcinoma was examined from this individual and in both cases, P-gp expression was detected in the cytoplasm of the malignant biliary structures as well as in the bile canaliculi of the normal surrounding tissue. These data suggests that these two tumor specimens from the same fish most probably were derived from a single malignant cholangiocyte which had elevated P-gp expression.

Immunohistochemical detection of cytochrome P450 1A expression

Cytochrome P450 1A expression was detected at relatively high levels in the hepatocytes of winter flounder non-tumorous liver tissue in 87% of tumor bearing Boston Harbor fish (7.5 ± 4.8 staining index), 100% of non-tumor bearing livers of Boston Harbor fish (11.1 ± 4.0 staining index), 100% of Cape Cod fish (10.5 ± 2.9 staining index) (Figure 2, Figure 4). CYP1A was detected at low to moderate levels in the two

cholangiocellular carcinomas from the sole fish which showed expression of P-gp in the tumor. No CYP1A staining was detected in hydropically vacuolated cells in liver of any fish. For all three sample groups, strong CYP1A staining was also detected in liver endothelium with levels in normal tissue of tumor bearing fish slightly (30%) depressed compared to that in specimens from Cape Cod Bay (Table 1). Low to moderate CYP1A staining was also detected in bile ducts and bile ductules (Table 1). CYP1A staining was significantly depressed in bile ducts in normal liver tissue of tumor bearing fish compared to fish from the reference site. And CYP1A levels were significantly elevated in bile ductules in non-tumor bearing fish from Boston Harbor compared to Cape Cod Bay fish.

Discussion

The immunohistochemical results establish that there are one or more proteins that cross-react with mAb C219 in the liver of winter flounder. Based on the known specificity of this antibody (Kartner *et al.*, 1985, Georges *et al.*, 1990), the proteins are likely P-gps. Based on the partial sequence of the two flounder P-gps (Chan *et al.*, 1992), mAb C219 cross-reacts with an epitope (Kartner *et al.*, 1985) predicted to be present in both proteins and thus we cannot distinguish between expression of isoform fpgpA responsible for bile acid export (Ballatori *et al.*, 2000) from isoform fpgpB the presumed multixenobiotic resistance protein (Cooper, 1996).

In contrast to expectations based on elevated P-gp levels being commonly observed within hepatocellular carcinomas in mammals (reviewed by Bradley and Ling, 1994) and fish (Köhler *et al.*, 1998, Cooper *et al.*, 1999), P-gp was highly expressed in the non-tumorous liver parenchyma surrounding the cholangiocellular carcinoma but was only detected within the carcinoma of one individual. The only published study of P-gp expression in mammalian cholangiocellular carcinomas showed elevated P-gp in the tumor itself in 8 of 12 human patients (Itsubo *et al.*, 1994). The expression of P-gp in

surrounding tissue was not reported in that study, and in fact is rarely reported in the literature.

In our study, the single individual which showed P-gp expression within the carcinoma, P-gp was observed in the cytoplasm of the malignant biliary structures. Detection of P-gp in the cytoplasm of fish tumor cells was previously reported in hepatocellular carcinomas from killifish (*Fundulus heteroclitus*) at a creosote contaminated site (Cooper *et al.*, 1999). As in our study, in those killifish P-gp expression in bile canaliculi in the parenchyma surrounding the tumor was elevated when compared to P-gp levels in bile canaliculi in non-tumor bearing fish from a reference site. In a study of hepatocellular carcinomas in the European flounder (*Platichthys flesus*), P-gp expression was observed to be greatest at the rims of carcinomas which were invasively expanding into extrafocal liver tissue (Köhler *et al.*, 1998). Thus, in contrast with our results for winter flounder showing lack of P-gp in advanced cholangiocellular carcinomas, in killifish and European flounder, elevated P-gp levels in hepatocellular carcinomas appear to be related to advanced tumor progression and resembles the classical multidrug resistance phenotype in mammalian hepatocellular tumors (Köhler *et al.*, 1998, Cooper *et al.*, 1999).

Our observation that CYP1A expression is low in the cholangiocellular carcinoma itself compared to the surrounding hepatocytes is similar to results showing depressed CYP1A in fish hepatocellular carcinomas (Van Veld *et al.*, 1992, Köhler *et al.*, 1998) and depressed total CYPs in mammalian hepatocellular carcinomas (el Mouelhi *et al.*, 1987, De Flora *et al.*, 1989, Habib *et al.*, 1994). This pattern of enzyme expression is one of the most consistent biochemical alterations in liver cancers (Habib *et al.*, 1994) and has been proposed by Farber and colleagues (Farber, 1984, Roomi *et al.*, 1985) to reflect a physiological response to hazardous chemicals by preneoplastic and neoplastic cells which thus become resistant to compounds such as PAHs that require activation by CYP1A to exert toxic effects. Although the levels of anthropogenic contaminants that are CYP1A

inducers, such as chlorinated aromatic hydrocarbons, are much lower in Cape Cod Bay fish than Boston Harbor fish (Moore *et al.*, 1996), levels appear to be sufficient to induce CYP1A in Cape Cod Bay fish to the same level as that observed in Boston Harbor fish. Similar elevated levels of CYP1A in the hepatocytes of normal liver tissue in tumor and non-tumor bearing fish from both sites suggests that fish from Cape Cod Bay and Boston Harbor may be maximally induced.

Previous work in mammalian *in vitro* systems has suggested that some CYP1A parent compounds or metabolites may induce P-gps (Gant *et al.*, 1991, Schrenk *et al.*, 1994, Fardel *et al.*, 1996). The lack of correlation between CYP1A and P-gp expression in the winter flounder populations we examined suggests that exposure to environmental contaminants, including CYP1A inducers, did not stimulate elevated P-gp in normal liver parenchyma of tumor bearing fish. Rather, it would appear that the presence of hepatic disease in these fish may be responsible for increased P-gp expression in hepatic parenchyma.

Two possible explanations for elevated P-gp in the parenchyma surrounding the cholangiocellular carcinomas are (i) that the tumor itself may be a source of stress to the liver that either directly or indirectly induces P-gp, perhaps via a response to cellular damage or (ii) that elevated P-gp transporters may increase the capacity for elimination of bile to compensate for ductules blocked with hydropically vacuolated cells and functionally unorganized malignant biliary structures. In the first case, there are abundant examples of mammalian studies showing that physiological perturbation in liver cells *in vitro* and *in vivo* can stimulate the expression of mdr genes. In rat primary hepatocyte cells overexpression of mdr1b was correlated with time in culture in the absence of drug exposure and presumably was a response by the hepatocytes to cellular stress of an unfamiliar environment stimulated by cell isolation and plating (Fardel *et al.*, 1992a, Fardel *et al.*, 1992b). Following partial hepatectomy in rats, mdr1a and mdr1b (Fairchild *et al.*,

1987, Thorgeirsson *et al.*, 1987, Teeter *et al.*, 1993, Nakatsukasa *et al.*, 1993) and mdr2 (Teeter *et al.*, 1993) were elevated in the regenerating liver. A gradient of mdr expression was observed in the liver acinus during regeneration with an increase (and highest levels) being first observed in zone 1 and gradually extending to zone 3 (Nakatsukasa *et al.*, 1993).

Tumors are known to elicit stress, such as hypoxia, in surrounding tissue which may lead to cellular damage of that adjacent tissue. Such cellular stresses are known to generate ceramides (Thévenod *et al.*, 2000), short chain lipids that stimulate the production of reactive oxygen species and initiate apoptosis (Green and Reed, 1998, Perry and Hannun, 1998), and activate the NF-κB transcription factor (Wiegmann *et al.*, 1994) which positively regulates mdr1 genes (Zhou and Kuo, 1997, Ogretmen and Safa, 1999). P-glycoproteins may play an anti-apoptotic role under conditions of physical stress by extruding ceramides (van Helvoort *et al.*, 1996, Thévenod *et al.*, 2000). The elevated P-gp levels observed in our study may be a defense mechanism on the part of bordering tissue to resist cellular damage stimulated by the invasive cholangiocellular carcinoma.

Alternatively, elevated P-gp in our study may be a response to the symptoms associated with the disease state of hydropic vacuolation combined with cholangiocellular carcinoma. The etiology of bile duct tumors in these flounder specimens has been previously described (Moore *et al.*, 1997). Contaminant exposure is intimately linked to the incidence of hydropic vacuolation which in turn is correlated with the occurrence of cholangiocellular carcinomas in these fish. Through an unknown mechanism, centrotubular preductular cells (the presumed fish equivalent of mammalian oval cells) swell with vacuoles containing water, electrolytes, and flocculent cellular debris (Moore *et al.*, 1997). Histological examination of the specimens used in our study shows that the canalicular microvilli are somewhat reduced, bile ductules and ducts become dilated and

hyperplastic and accumulate bile fluids within them (Moore *et al.*, 1997). The histological data suggest that hydropic vacuolation may impede normal bile flow possibly producing a mild cholestatic state. However, the lack of classical ultrastructural signs of this disease (Phillips *et al.*, 1987) such as dilated canaliculi that have lost their microvillar border and weep granular and vesicular secretions in these specimens (Moore *et al.*, 1997) suggests that the condition has not progressed to a severe cholestatic state. The inability of a hydropically vacuolated ductules to efficiently conduct bile may stimulate the proliferation of bile duct structures.

These proliferating biliary structures are unorganized and may not increase bile elimination and thus not ameliorate the original problem of accumulation of bile constituents in hepatocytes. Another way to increase bile elimination would be to increase the level of transporters of bile constituents in the bile canaliculi. Elevated P-gp in normal non-tumor liver parenchyma surrounding cholangiocellular carcinomas may be such a response to the inefficient elimination of bile in these diseased livers. The blockage of bile ductules due to hydropic vacuolation would cause the regurgitation of bile constituents into the hepatocytes, a condition which has been previously shown to induce mdr genes in rodent models (Phillips *et al.*, 1986). Both drug-induced (Schrenk *et al.*, 1993, Schrenk *et al.*, 1993) and obstructive (bile duct ligation) (Schrenk *et al.*, 1993, Accatino *et al.*, 1996, Kagawa *et al.*, 1998) biliary cholestasis in mammals is associated with elevated expression of mdr genes and P-gps. We speculate from our results that P-gps may be induced not only by severe short-term cholestasis but perhaps also by mild chronic cholestasis. In our flounder specimens, elevated P-gp may be a hepatoprotective response to stimulate elimination of accumulated cytotoxic bile constituents. We cannot further test these hypotheses in this feral flounder population because there has been a dramatic decline in the prevalence of neoplasms since these specimens were collected, correlated with a reduced chemical input at the Deer Island outfall in Boston Harbor (Moore *et al.*, 1996).

Conclusion

P-gp expression in cholangiocellular carcinomas of winter flounder (*Pleuronectes americanus*) from a highly contaminated site in Boston Harbor, Massachusetts do not exhibit the classical multidrug resistance phenotype commonly observed in hepatocellular carcinomas in both fish and mammals. In contrast to expectations, we observed a general lack of P-gp expression in cholangiocellular carcinomas and associated vacuolated cells of winter flounder while P-gp was highly expressed in bile canaliculi of non-tumorous liver parenchyma surrounding the cholangiocellular carcinoma. The lack of correlation between CYP1A and P-gp expression in the winter flounder populations we examined suggests that exposure to environmental contaminants in Boston Harbor, including CYP1A inducers, did not stimulate elevated P-gp in normal liver parenchyma of tumor bearing fish. Rather, it would appear that the presence of hepatic disease in these fish may be responsible for increased P-gp expression in hepatic parenchyma. We suggest that cellular stress caused by impaired bile elimination, possibly linked to the presence of the cholangiocellular carcinomas in these fish, may be responsible for the elevated P-gp observed in the normal liver parenchyma surrounding tumors.

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Cytochrome P450 1A   P-glycoprotein

Figure 1. Immunohistochemical images of cytochrome P450 1A by mAb 1-12-3 (left column) and P-glycoprotein by mAb C219 (right column) in tumor bearing winter flounder livers from Boston Harbor. Sample identification is as follows: cholangiocellular carcinoma (A & B); hepatocytes adjacent to tumor (C & D); hydropically vacuolated cells (E & F).
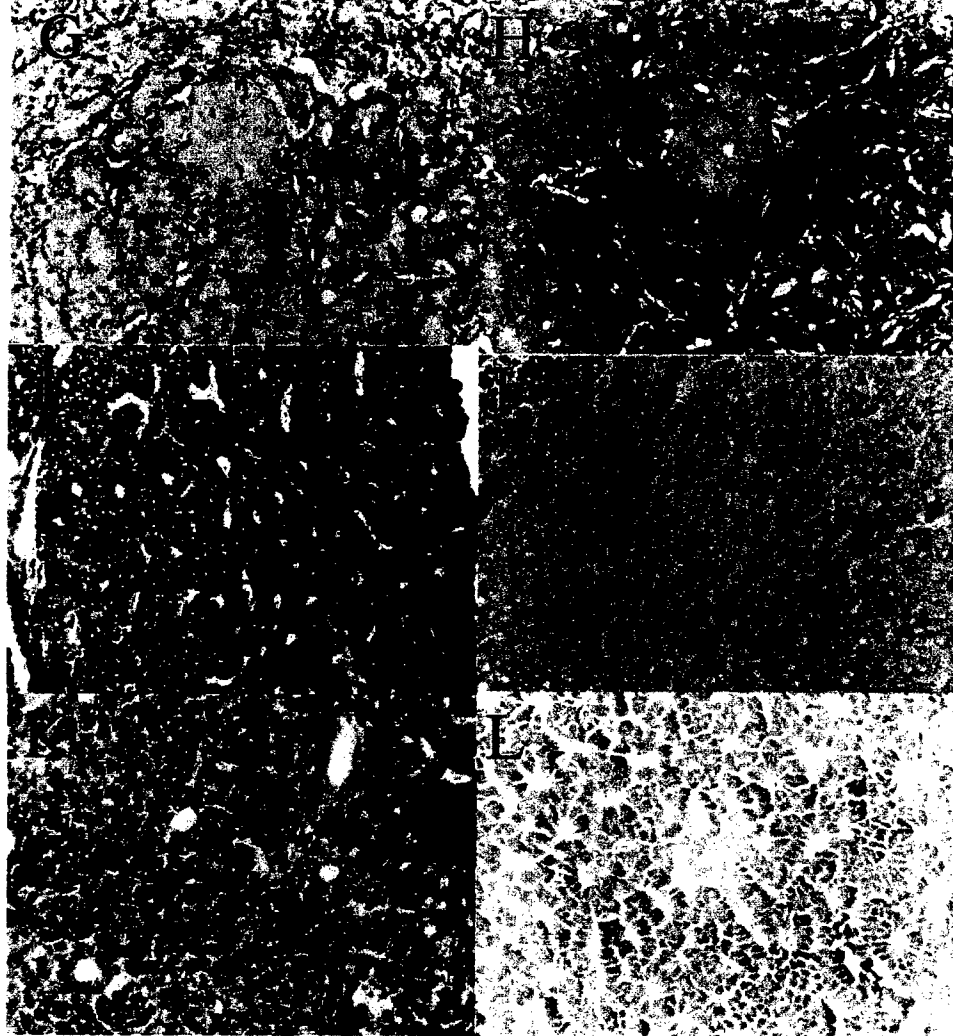
Cytochrome P450 1A   P-glycoprotein

Figure 2. Immunohistochemical images of
cytochrome P450 1A by mAb 1-12-3 (left column)
and P-glycoprotein by mAb C219 (right column) in
winter flounder livers. Sample identification is as
follows: postive staining cholangiocellular
carcinoma from Boston Harbor (G & H); liver from
non-tumor bearing flounder from Boston Harbor (I &
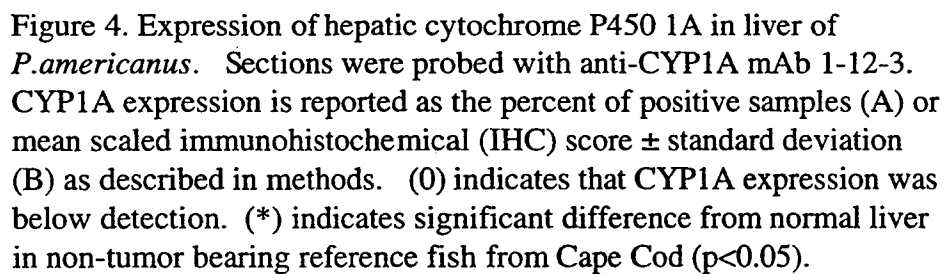J); liver from non-tumor bearing flounder from Cape
Cod Bay (K & L).

A

B

Tissue type:	normal liver	normal liver	hydropic vacuoles	cholangiocellular carcinoma	surrounding parenchyma
Site:	reference	Boston Harbor		Boston Harbor	
Sample size:	10	7		14	
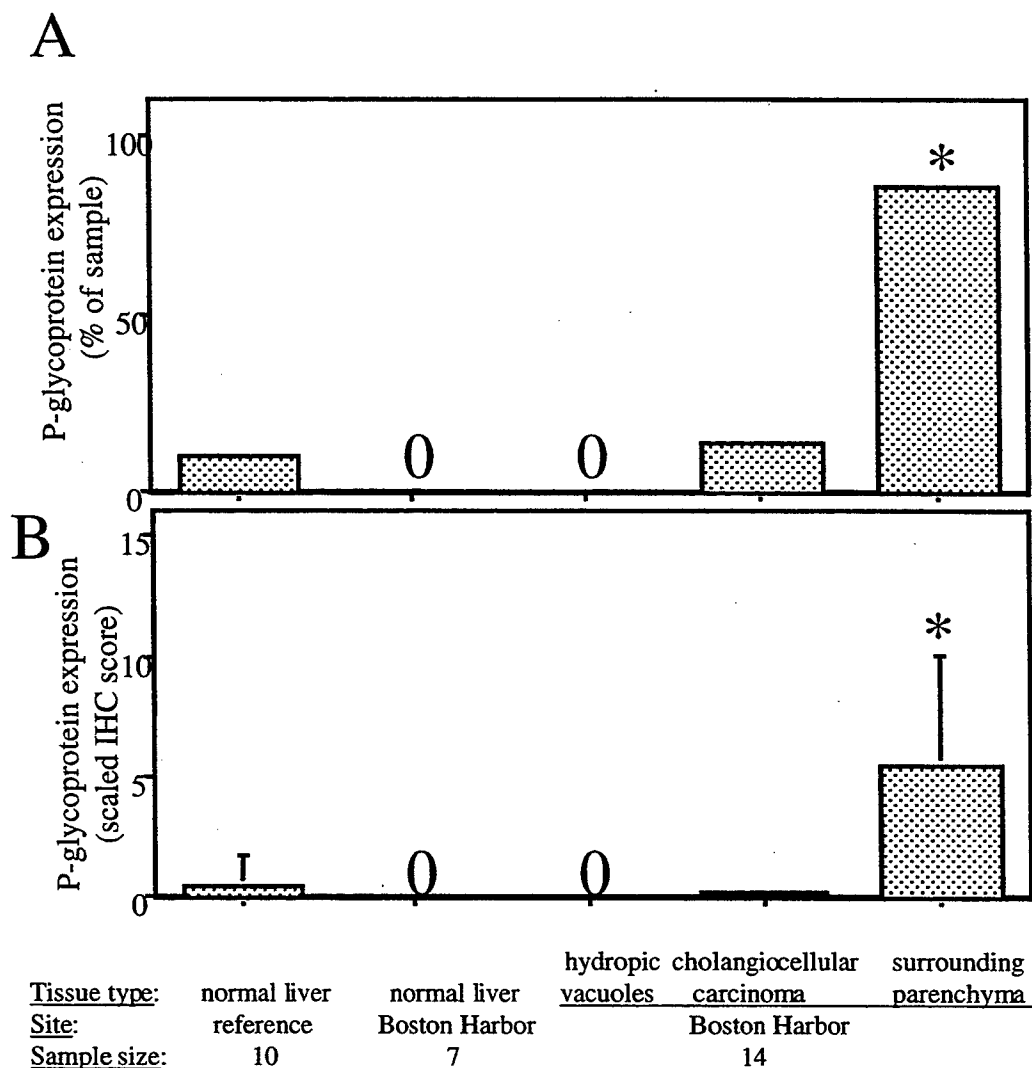
Figure 3. Expression of P-glycoprotein in liver of *P.americanus*. P-glycoprotein was localized to the bile canaliculi in all samples except for the two P-gp positive tumors which showed cytoplasmic P-gpexpression. Sections were probed with anti-Pgp mAb C219. P-gp expression is reported as percent of positive samples (A) or mean scaled immunohistochemical (IHC) score ± standard devitaion (B) as described in methods. (0) indicates that P-gp expression was below detection. (*) indicates significant difference from non-tumor bearing reference fish from Cape Cod (p<0.05).

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A

CYP1A expression
(% of sample)

100

50

0

*
0

*

B

15

CYP1Aexpression
(scaled IHC score)

10

5

0

*
0

*

	hydropic	cholangiocellular	surrounding
Tissue type: normal liver	normal liver	vacuoles	carcinoma	parenchyma
Site: reference	Boston Harbor		Boston Harbor	
Sample size: 10	7		14	

Figure 4. Expression of hepatic cytochrome P450 1A in liver of
P.americanus.   Sections were probed with anti-CYP1A mAb 1-12-3.
CYP1A expression is reported as the percent of positive samples (A) or
mean scaled immunohistochemical (IHC) score ± standard deviation
(B) as described in methods.   (0) indicates that CYP1A expression was
below detection. (*) indicates significant difference from normal liver
in non-tumor bearing reference fish from Cape Cod ($p<0.05$).

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site	tumors	hepatocytes	bile ductule	bile duct	endothelium
Cape Cod Bay	no	10.5 ± 1.9 (10)	1.5 ± 2.1 (9)	2.7 ± 2.2 (10)	12.8 ± 1.7 (9)
Boston Harbor	no	11.1 ± 4.0 (7)	5.7 ± 2.2* (5)	4.9 ± 1.4 (4)	12.9 ± 2.5 (5)
Boston Harbor	yes	7.5 ± 4.8 (16)	0.1 ± 0.2 (5)	0.6 ±1.1* (5)	9.2 ±3.1* (9)

Table 1. Cytochrome P450 1A expression in various liver cell types in *P.americanus*. Sections were probed with anti-CYP1A mAb 1-12-3. Values are reported as mean scaled immunohistochemical score ± standard deviation. Cell types other than hepatocytes were not present in every sample and the value in parentheses indicates the number of specimens in which each cell type was observed. * indicates significant difference from normal liver in non-tumor bearing reference fish from Cape Cod ($p < 0.05$).

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Chapter 4

Induction of P-glycoprotein and cytochrome P450 1A in fish (*Anoplarchus purpurescens*) exposed to environmental xenobiotics.

Abstract

Whether P-glycoproteins (P-gps) which confer multidrug resistance in tumor cell lines are important in adaptation to chemicals in natural populations of vertebrates exposed to contaminants is the focus of this study. We investigated whether levels of P-gp expression in the intertidal fish high cockscomb blenny (*Anoplarchus purpurescens*), might be altered by field and/or laboratory exposures to crude oil or pulp mill effluent, and whether P-gp expression might be related to cytochrome P450 1A (CYP1A) induction. In oil exposed fish, P-glycoprotein expression in the bile canaliculi increased 3- to 5-fold compared to expression levels in control fish. Levels of P-gp expression were highly correlated with hepatic CYP1A levels in these fish. In fish freshly caught from sites surrounding pulp mills, hepatic P-gp expression was 14-fold higher than in fish depurated in clean water for 6 weeks. However, P-gp expression in freshly caught fish did not correlate to proximity to pulp mills. CYP1A levels were elevated in hepatocytes of freshly caught as compared to depurated fish. Neither CYP1A nor P-gp were elevated in depurated fish exposed to sediment and food from within the original pulp mill effluent stream. Depurated fish which were injected with the aryl hydrocarbon agonist ß-naphthoflavone (BNF) showed an expected induction of CYP1A but no induction of P-gp. These results suggest that in blennies P-gp expression is not regulated by the aryl hydrocarbon receptor pathway, although P-gp and CYP1A may both be induced in blennies by some compounds in petroleum and unidentified xenobiotics at field sites.

While our data indicate that CYP1A and P-gp are not coordinately regulated, these proteins may play complementary roles in cellular detoxification. Thus P-gp activity may be an important mechanism of multixenobiotic resistance for organisms, such as intertidal fish, which are commonly exposed to anthropogenic contaminants and naturally occurring toxins.

Keywords: multixenobiotic resistance, multidrug resistance, P-glycoprotein, cytochrome P450 1A, marine environment, fish

Introduction

P-glycoproteins (P-gps) have been known for years to confer certain multidrug resistance phenotypes to tumor cell lines (Juliano and Ling, 1976) and tumors of human patients (Gerlach et al., 1986). Whether P-gps are important in adaptation to chemicals in natural populations exposed to contaminants is under investigation in this study. P-glycoproteins function as energy dependent efflux flippases that prevent the cellular accumulation of a wide variety of moderately hydrophobic compounds. Known P-gp substrates include endogenous regulators such as glucocorticoids (Naito et al., 1989; Ueda et al., 1992), drugs and other natural products (Gottesman and Pastan, 1988), and environmental contaminants (Phang et al., 1993; Cornwall et al., 1995; Bain and LeBlanc, 1996). P-glycoproteins can be induced by exposure to compounds of both natural and anthropogenic origin (Gant et al., 1991; Fardel et al., 1996; Schrenk et al., 1994).

Coastal pollutants such as crude oil and pulp mill effluent are composed of numerous chemicals but whether any of these compounds are P-gp inducers is unknown. Candidate P-gp inducers present in these contaminant mixtures are known to induce the biotransformation enzyme cytochrome P450 1A (CYP1A) including polycyclic aromatic hydrocarbons (PAH) (James and Bend, 1980; Lindstrom-Seppa, 1988), polychlorinated

biphenyls (Gooch *et al.*, 1989), aromatic amines (Ayrton *et al.*, 1990), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Janz and Metcalfe, 1991). CYP1A catalyzes the oxidation of some lipophilic pollutants which renders them more polar and hence more readily eliminated from the body by efflux transporters such as P-gps. Whether P-gps play a more important role in the transport of CYP1A metabolites rather than parent compounds has not been entirely elucidated (Phang *et al.*, 1993; Bain and LeBlanc, 1996). In mammalian *in vitro* systems exposure to some CYP1A inducers and substrates including benzo[a]pyrene (Fardel *et al.*, 1996), 3-methylcholanthrene (Gant *et al.*, 1991; Fardel *et al.*, 1996), and 2-acetylaminofluorene (2-AAF) (Schrenk *et al.*, 1994) has been reported to induce P-gps. TCDD-treated rats demonstrated coinduction of hepatic P-gp and CYP1A2 (Burt and Thorgeirsson, 1988) but similar exposures in mice did not produce increased P-gp levels, suggesting that transporter gene induction does not occur via the aryl hydrocarbon receptor (Ahr) pathway (Teeter *et al.*, 1991). Whether P-gps are induced by the parent compound or by CYP metabolites has not been adequately determined.

In the present study, our objectives were to determine whether levels of P-gp expression might be related to CYP1A induction in a natural population of vertebrates. We selected the high cockscomb blenny (*Anoplarchus purpurescens*) as the model organism because they are abundant and readily obtainable from the Pacific intertidal zone, are able to survive at polluted sites, and exhibit a very limited home range (approximately 15 m), thus reflecting exposure to xenobiotics at the collection site. We examined blennies exposed to pulp mill effluent or crude oil. The induction of CYP1A in blennies exposed to pulp mill effluent or petroleum has been extensively documented (Kantoniemi *et al.*, 1996; Woodin *et al.*, 1997). We also examined the cellular localization of CYP1A and P-gp expression in various organs of exposed fish. Whether P-gps, which are expressed in many tissues of teleost fish (Hemmer *et al.*, 1995) which also express CYP1A (Smolowitz *et al.*, 1991),

are induced by these same exposures is not known. Determining whether P-gp levels are elevated in wild populations exposed to such pollutants, and its relationship to CYP1A expression, will help us to better understand the biochemical mechanisms employed to permit animals to survive in environments containing both naturally occurring toxins and anthropogenic contaminants.

Methods

British Columbia Field Collections

High cockscomb blennies (*A. purpurescens*) were collected by hand from beneath stones in the mid-intertidal zone exposed during low tide. Five to six fish were obtained from each of 3 field sites at different distances downstream from pulp mills in Georgia Strait, British Columbia, Canada (Figure 1). No blennies were found at beaches within a 13 km radius of a pulp mill. Blennies were sampled from Porteau Cove (13 km from the nearest pulp mill), Lions Bay (22 km), and a reference site at Hornby Island (40 km) (July 13-16, 1996). Blennies were sacrificed in the field by cervical scission, the abdominal wall was cut along the ventral midline from anus to gill operculum, and carcasses were stored in 10% neutral buffered formalin for several months. Upon return to the laboratory, the liver, kidney, gill, nongravid gonad (sex determination was not possible), heart, spleen, and gastrointestinal tract were excised from the carcasses and embedded in paraffin for immunohistochemical examination.

At the time of sampling, fishing closures were in effect at Porteau Cove and Lions Bay due to organochlorine contamination from pulp mill effluent (Department of Fisheries and Oceans Canada, 1995; Department of Fisheries and Oceans Canada, 1996). Evaluation of mid-low intertidal diversity during a 1997 survey demonstrated depletions at Porteau (16 species) and Lions Bay (29 species) compared to the reference site on Hornby Island (80 species) (biodiversity data not shown). Since 1990, low diversity of intertidal species has

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been documented at Porteau and Lions Bay compared to the Hornby Island reference site (Bard, 1998). Although Hornby is not contaminated with organochlorines from mill effluent, this site is exposed to other sources of anthropogenic pollution including leakage from septic tanks, combustion by-products, and fuel from ferry and pleasure boats.

Amphipods (family Grammarus) and isopods (*Idotea wosnesenskiis* and *Gnorimosphaeroma oregonensis*) which comprise a major food source for blennies (as determined by examination of gut contents of dissected blennies) along with sediment and wood fibres were collected from Darrel Bay on June 13, 1996. Darrel Bay is situated 6 km downstream from the Woodfibre pulp mill effluent diffuser. These samples were shipped on ice to Woods Hole Oceanographic Institution, Woods Hole, MA where they were stored sealed at 4°C for 6 weeks prior to use.

A group of live fish were collected from Hornby Island on July 30, 1996 and were transported to Woods Hole. Blennies were maintained in flowing, filtered seawater at 15° C with aeration and were fed daily with previously frozen brine shrimp.

Twelve high cockscomb blennies were collected from Lions Bay on August 22, 1998. Within hours of collection, liver, kidney, gill, nongravid gonad (sex identification was not possible), heart, spleen, and intestine were dissected. Samples were flash frozen in liquid nitrogen, express shipped on dry ice to Woods Hole, and transferred to –70° C freezer for storage until Western blot analysis was completed.

ß-Naphthoflavone Treatments and Effluent Exposed Sediment and Food.

Blennies collected from the reference site at Hornby Island were held in clean aquaria at Woods Hole for 30-43 days. Five to six blennies were then exposed to one of three treatment conditions: ß-naphthoflavone (BNF), a model CYP1A inducer; the corn oil vehicle (control); or pulp mill effluent exposed sediment and food. Fish were given intraperitoneal (i.p.) injections on days 0 and 3 of BNF in corn oil, at a dose of 10 mg of

BNF per kilogram of body weight at each injection or with an equivalent volume of corn oil (10 μL per gram of body weight). This BNF dosing regimen has been previously found to produce a strong CYP1A response in fish Kloepper-Sams and Stegeman, 1992. Blennies in the third treatment group were maintained for 16 days over sediment and wood fibres and fed isopods and amphipods collected from Darrel Bay, Canada, a site within the pulp mill effluent stream. Treated fish were killed by cervical scission on day 6 (BNF exposure experiment) or day 16 (sediment and food exposure experiment). Portions of livers were reserved for ethoxyresorufin-O-deethylase (EROD) activity analysis while the remaining liver, kidney, gill, nongravid gonad, heart, spleen, and gastrointestinal tract were fixed and stored in 10% neutral buffered formalin for several months and embedded in paraffin for immunohistochemical analysis.

Archived Alaskan Field Collections and Laboratory Exposure to Oil

In our study, we examined archived samples which had been previously obtained (Woodin *et al.*, 1997). In the original study, live high cockscomb blennies were collected from Hinchinbrook Island, a putative clean field site in Prince William Sound, Alaska (June 15-25, 1990), transported to Woods Hole, and depurated for 6 months in clean aquaria as previously described (Woodin *et al.*, 1997). During the same period, sediment and amphipods (family Grammarus) were collected from Knight Island, AK, a site oiled by the Exxon Valdez spill (March 24, 1989). Depurated fish were divided into four groups (5 fish per group) and were exposed for 3 weeks to either: clean food and clean sediment; clean food and oiled sediment; oiled food and clean sediment; or oiled food and oiled sediment as previously described (Woodin *et al.*, 1997).

Western Blot for P-glycoprotein

Processing of liver, kidney, gill, nongravid gonad, heart, spleen, and intestine (divided into foregut and hind gut) from 12 blennies freshly collected from Lions Bay, Canada and subsequent immunoblot analysis for P-gp expression followed the protocols previously described (Cooper et al., 1996) using the affinity purified murine monoclonal antibody (mAb) C219 (IgG$_{2a}$) (Signet Laboratories, Inc., Dedham, MA) which is immunoreactive against the mammalian P-gp (Kartner et al., 1985). C219 recognizes an internal, highly conserved amino acid sequence common to human MDR1 and MDR2(3) of the 170 kDa transmembrane glycoprotein, as well as the sister of P-glycoprotein (spgp) also known as the bile salt export pump (BSEP), and all other mammalian and non-mammalian mdr isoforms whose sequence is known (Kartner et al., 1985; Endicott and Ling, 1989; Georges et al., 1990).

Tissues from 6 individuals were combined together to form 2 pools for each of the following organs: kidney, nongravid gonad, gill, and brain. Tissues from all 12 individuals were combined to form a single pool for the following organs: spleen, fore gut, and hind gut. Five livers was examined separately. The protein concentrations of tissue lysates were determined using the bicinchoninic acid (BCA) procedure (Smith et al., 1985). Tissue lysates were diluted to 50 µg total protein with standard SDS-PAGE sample buffer, denatured (65° C, 4 min), and loaded onto a 4-12% tris glycine gel (Novex, San Diego, CA). Pooled killifish (Fundulus heteroclitus) liver lysates were used as positive P-gp controls (Cooper et al., 1996). Electrophoresis and electrotransfer to nitrocellulose membranes followed the manufacturer's recommendations (Novex, San Diego, CA). Transfer efficiency and protein loading consistency was check by Ponceau staining the nitrocellulose. Membranes were blocked in 5% (w/v) non-fat dry milk in TTBS 9100 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.02% Tween 20) and incubated in mAb C219 for 1 hour (Signet, Dedham, MA) (2.5 µg/ml in TTBS with 1% (w/v) non-fat dry milk).

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Membrane was washed 3 times with TTBS and incubated with secondary antibody (SAM-HRP sheep anti-mouse IgG horseradish peroxidase (diluted 1:1000 with TBS, 5% (w/v) non-fat dry milk (Amersham Life Sciences, Little Chalfont, England). Blot was incubated with ECL reagents luminol and P-coumerin hydrogen peroxide and then pressed against autoRad X-ray film as previously described (Matthews *et al.*, 1985).

Developed film was photographed with a digital camera and densitometry measurements were taken using the NIH computer program in order to estimate relative P-gp content in liver extracts. P-glycoprotein content in each sample was expressed in arbitrary units made by assigning a value of 100 to the signal obtained for 5 μg of pooled *F. heteroclitus* liver lysate. *F. heteroclitus* liver was selected as a positive control because P-gp has previously been detected by Western blot in such samples (Cooper *et al.*, 1996).

Immunohistochemical analysis
Cytochrome P450 1A detection

Paraffin-embedded samples were sectioned at 5μm on Super-frost plus slides, deparaffinated, hydrated, and immunochemically stained using an indirect peroxidase stain (Universal Immunoperoxidase Staining Kit (Murine), Signet Laboratories, Inc., Dedham, MA) with mAb 1-12-3 to scup cytochrome P4501A as the primary antibody, as described previously (Smolowitz *et al.*, 1991). Previous immunofluorescence studies have demonstrated the specificity of mAb 1-12-3 for P4501A in tissue sections by immunoadsorption (Miller *et al.*, 1988).

P-glycoprotein detection

Procedures were similar to cytochrome P450 1A detection except for the following changes which were adapted from Hemmer et al. (1995). Sections were mounted on poly-L-lysine-coated slides (Sigma, St. Louis, MO), deparaffined, and then hydrated with 0.5%

bovine serum albumin/0.5% nonfat dry milk in TPBS (0.05% Tween 20 in PBS). Monoclonal antibody C219 served as the primary antibody (10 μg/ml in TPBS).

Stain quantification

Specific staining by mAb 1-12-3 or mAb C219 was evaluated by light microscopic examination of the stained sections. Cell types that stained and their associated occurrence and staining intensity were recorded for each tissue section examined. Staining results were recorded as intensity in any one cell type: negative (0), mild (1), mild-to-moderate (2), moderate (3), strong (4) or very strong (5); and as degree of occurrence in any one cell type: absent (0), rare (1), some (1.5), many (2) and all (3). Quantitative comparisons were made between tissue types at various sites by using the product of intensity and occurrence, giving a range of score from 0 to 15. This scoring scheme has been described previously (Woodin *et al.*, 1997).

Ethoxyresorufin-O-deethylase (EROD) activity assays

Livers were homogenized and centrifuged as previously described (Stegeman *et al.*, 1979) to yield microsomes. Nine to ten blennies were examined from each of the three post-depuration laboratory treatments on British Columbia fish: BNF; control corn oil; and pulp mill effluent exposed diet and sediment. The protein concentrations of microsomal resuspensions were determined using the bicinchoninic acid (BCA) procedure (Smith *et al.*, 1985). EROD assays were performed in multi-well plates using a cytofluor plate reader to measure cytochrome P450 1A activity as previously described (Hahn *et al.*, 1996).

Statistical Analyses

Differences between reference and field sites and between control and treatment group means were statistically analyzed by one-way ANOVA using Dunnett's one-tailed procedure for unequal sample sizes using the SuperANOVA (Abacus Concepts) statistical

program; p≤ 0.05 was accepted as significant. For the oil exposure experiment, the same program was used to perform linear regression analysis between the means of CYP1A expression in hepatocytes (previously reported by Woodin et al. (1997)) and the means of P-gp expression in bile canaliculi as determined by IHC analysis; p≤ 0.05 was accepted as significant.

Results

Western blot detection of P-glycoprotein in freshly caught fish

Initially we examined lysates from multiple organs of freshly caught *A. purpurescens* from Lions Bay, Canada, a site moderately exposed to pulp mill effluent. Western blot showed the presence of proteins which cross-react with the mammalian anti-P-gp mAb C219, confirming the utility of this antibody for detecting P-gp in blennies. Monoclonal antibody C219 was immunoreactive with a single diffuse band at approximately 170 kDa for liver lysates, but immunoreactivity was below detection in kidney, gill, fore gut, hind gut, nongravid gonad, brain and spleen lysates (Figure 2). Large interindividual variability of hepatic P-gp expression was detected (19 ± 14 relative units of integrated density).

Pulp mill effluent field exposures and laboratory treatments

Immunohistochemical detection of P-glycoprotein expression

In order to determine the cellular localization of P-gp, paraffin embedded fixed tissue sections were probed with mAb C219. In *A. purpurescens* collected from Canadian field sites, P-glycoprotein expression was localized to the bile canaliculi in liver (Figure 3). P-glycoprotein was not detected by immunohistochemistry in other organs sampled, which included kidney, gill, gonad, gastrointestinal tract, spleen, and heart. Hepatic P-gp staining was significantly elevated >14-fold in fish from all three field sites compared to

control fish which had been depurated for >4 weeks in clean tanks (Figure 4). However, hepatic P-gp expression was not significantly different between field sites and did not correlate to level of exposure to pulp mill effluent.

Blennies fed a diet of isopods and amphipods collected from Darrel Bay and exposed to sediment from this field site which is highly exposed to pulp mill effluent, had no elevated hepatic P-gp compared to depurated fish (Figure 4). Blennies exposed to BNF, a model CYP1A inducer, also showed no induction of hepatic P-gp.

Cytochrome P450 1A catalytic function

As expected, ethoxyresorufin-O-deethylase activity assays on hepatic microsomes demonstrated significantly elevated CYP1A catalytic function in blennies exposed to BNF (>34-fold) compared to corn oil controls (Figure 5). Fish exposed to sediment and food collected from Darrel Bay, a field site within the pulp mill effluent stream, did not have elevated EROD activity compared to corn oil controls (Figure 5).

Immunohistochemical detection of Cytochrome P450 1A expression

CYP1A staining indices were similar in hepatocytes of fish freshly caught from Lions Bay, Porteau Cove, and Hornby Island and BNF-exposed fish. No CYP1A expression was detected in hepatocytes of depurated control fish (Figure 3, Figure 4, and Table 1). Depurated fish subsequently exposed to sediment and food collected from Darrel Bay, a field site within the pulp mill effluent stream, also had no detectable expression of CYP1A in hepatocytes. CYP1A was significantly induced in BNF-treated fish in all tissues and cell types examined except for kidney tubules and stomach epithelium in which CYP1A was present but staining indices were not significantly elevated compared to levels in these cell types in control blennies (Table 1). The only cell type examined in which CYP1A was not detected in BNF-treated blennies was in spleen parenchyma. In the gonad

vascular endothelial cells, CYP1A staining was very strong in BNF-treated fish and not detected in fish of any other treatment group. For all other treatment and field conditions (not including BNF), CYP1A staining in gill, kidney, and heart was detected at similar levels to that in control fish. CYP1A was detected in the gastrointestinal tract only in freshly caught and BNF-treated fish.

Comparison of hepatic P-gp and CYP1A levels in freshly caught fish.

A simple regression showed a moderate positive relationship between hepatic P-gp and CYP1A expression ($r^2= 0.496$, $p<0.0001$) for all freshly collected and lab maintained fish excluding BNF-treated fish. Large interindividual variability was observed in the level of immunostaining of both P-gp and CYP1A in livers of freshly caught fish. Seventy-three percent of all the freshly caught blennies from the three British Columbia sites were observed to have hepatic expression of both P-gp and CYP1A. Twenty percent of these fish displayed only induction of P-gp and no detectable expression of CYP1A. One fish of the 15 examined had neither measurable hepatic P-gp nor CYP1A expression.

P-glycoprotein in fish exposed to oiled food and sediment

Archived samples of *A. purpurescens* that had been exposed to petroleum were sectioned and stained with anti-P-gp mAb C219. P-glycoprotein was localized to the bile canaliculi in liver but was not detected by immunohistochemical analysis in any of the other examined organs: kidney, gill, gonad, gastrointestinal tract, spleen, and heart. P-glycoprotein expression in the bile canaliculi increased from 3- to 5-fold in blennies exposed to oiled food and/or oiled sediment, compared to blennies held over clean sediment and fed uncontaminated food (Figure 6). However, only the exposure to clean food and oiled sediment produced statistically significant increases in hepatic P-gp expression compared to levels in control fish.

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These same samples were previously examined for hepatic CYP1A expression (Woodin *et al.*, 1997). The results for CYP1A were similar to the results here for P-gp: hepatocyte CYP1A expression increased 4- to 17-fold in blennies that were exposed to oiled food and/or oiled sediment, and only the clean food/oiled sediment treatment produced statistically significant induction of hepatic CYP1A expression compared to control treatment (Woodin *et al.*, 1997). The expression of P-gp in bile canaliculi is correlated to the expression of CYP1A in hepatocytes of blennies exposed to oil (r^2 =0.919) (Figure 7).

As with P-gp in freshly collected fish from British Columbia (Porteau Cove, Lions Bay, and Hornby Island), there was substantial interindividual variability in the pattern of induction of P-gp and CYP1A in the fish exposed to oil. Within the control group (N=5), 60% of fish had detectable levels of P-gp but no CYP1A observed, 20% had CYP1A detected but not P-gp, and 20% had no induction of either protein. In blennies exposed to oiled food and/or oiled sediment, 86% had induction of both P-gp and CYP1A while one fish had induction of P-gp and not CYP1A and another showed CYP1A but not P-gp staining (Woodin *et al.*, 1997). These observations suggest that there is a low level of P-gp expression in the majority of control fish even in the absence of CYP1A induction and that there is a parallel increase in levels of P-gp and CYP1A in the majority of fish exposed to some level of oil contamination.

Discussion

In this study, intertidal fish *A. purpurescens* were exposed to common marine pollutants (pulp mill effluent or crude oil) in the field and/or in the laboratory. Our objectives were to determine if P-gp expression is induced by the same exposures that

induce CYP1A and to compare cellular localization of these two proteins in various organs of exposed fish.

Pulp Mill Exposures

The immunoblot results establish that there is a protein present in the liver of *A. purpurescens* that is cross-reactive with mAb C219. Based on the known specificity of this antibody (Kartner *et al.*, 1985; Georges *et al.*, 1990), and the fact that the approximate molecular weight of the immunoreactive band (170 kDa) is in the known range for P-gps (Kartner *et al.*, 1985; Georges *et al.*, 1990), we concluded that C219 detects P-gp in blenny. The immunohistochemical results using the same antibody validly reveal P-gp to be localized to the bile canaliculi in *A. purpurescens*. P-glycoprotein was expressed in liver of depurated fish at levels just above the limit of detection for our immunohistochemical protocol. Recent studies in our laboratory have demonstrated that the limit of detection can be lowered by using a more sensitive protocol that involves a shorter time for tissue fixation, use of an unmasking agent such as Retrieve-All (Signet, Dedham, MA) and a biotin based detection system such as USA Ultra Streptavidin detection kit (Signet, Dedham, MA) during immunohistochemical staining procedures (results not shown).

Both hepatic CYP1A and P-gp expression levels were elevated in freshly caught fish from each of three British Columbia field sites compared to fish originally collected from Hornby Island that had been depurated in clean aquaria. The decline of P-gp levels in depurated blennies implies that P-gp expression, as with CYP1A (Woodin *et al.*, 1997), may be induced in response to environmental xenobiotics that blennies are exposed to via sediment, water, or diet. The >14 fold increase in hepatic P-gp levels of freshly collected fish versus depurated fish is a similar order of increase as that previously reported in livers of mammals exposed to drugs *in vivo*: rats exposed to 2-acetylaminofluorene or 3-methylcholanthrene for 36 hours showed a 5-fold increase in P-gp (Gant *et al.*, 1991);

monkeys had up to a 15-fold, 13-fold, and 6.5-fold increase in P-gp upon a 7 day exposure to respectively either erythromycin, rifampicin, or tamoxifen (Gant *et al.*, 1995).

Hepatic P-gp expression in freshly caught fish from all sites, as with CYP1A expression, did not significantly differ between sites and was not related to proximity to nearest pulp mill. This lack of correlation suggests that constituents of pulp mill effluent are not solely responsible for elevated P-gp and CYP1A expression. Conceivably, at each site a variety of different compounds from anthropogenic and/or natural sources could have the capacity to induce P-gp and/or CYP1A. For example, although the reference site on Hornby Island is not exposed to pulp mill effluent, other sources of anthropogenic pollutants (e.g. septic tank leakage, fuel from recreational boat and ferry traffic) and natural products (e.g. toxins from algal blooms) are present and may contribute to P-gp and/or CYP induction. Similarly the elevated levels of P-gp and CYP in fish from sites close to the pulp mill might be due to effluent exposure and/or to unknown sources of inducers. The similar levels of both hepatic P-gp and CYP1A in fish from all three field sites suggests that similar levels of inducers of unknown source were present at each location.

Whether the same environmental compounds act to induce both P-gp and CYP1A is not known. However, two observations suggest that P-gp and CYP1A are not coordinately regulated in these fish. First, elevated levels of P-gp and CYP1A occur in different cellular locations in liver: P-gp expression was restricted to hepatocyte margins forming the bile canaliculus while CYP1A is found throughout the cytoplasm of hepatic parenchymal cells (as well as in vascular endothelium and bile ducts). Second, although the expression levels of hepatic P-gp and CYP1A were correlated in the majority of freshly caught fish from all three field sites, twenty percent of these fish demonstrated elevated P-gp expression without any detectable expression of CYP1A.

In order to test whether the elevated levels of P-gp in freshly caught fish could be the result of exposure to CYP1A inducers, we exposed depurated fish that were originally

collected from Hornby Island to BNF, a model CYP1A inducer and aryl hydrocarbon receptor agonist. The magnitude of hepatic CYP1A in freshly caught *A. purpurescens* was similar to levels observed in fish exposed to BNF in the laboratory. However, BNF did not induce P-gp in blennies. Early studies of TCDD-treated rats reported coinduction of hepatic *mdr* and CYP1A2 mRNA suggesting an aryl hydrocarbon-receptor-mediated induction of P-gp (Burt and Thorgeirsson, 1988). Subsequent work has not supported this claim: similar exposures in mice have not detected increased P-gp levels and the researchers concluded that mdr gene induction does not occur via the aryl hydrocarbon pathway (Teeter *et al.*, 1991). The lack of P-gp increase in BNF-treated fish suggests that BNF may not yield a metabolite that is a P-gp substrate. A similar lack of P-gp induction in fish treated with a CYP1A inducer was observed in killifish (*Fundulus heteroclitus*) exposed by i.p. injection to 3-methylcholanthrene (3-MC), a model PAH (Cooper *et al.*, 1999). 3-methylcholanthrene had been previously reported to induce P-gp as well as CYP1A1 and CYP1A2 in primary rat hepatocyte cultures (Gant *et al.*, 1991). In contrast, studies of nonparenchymal rat liver epithelial (RLE) cells treated with 3-MC demonstrated P-gp induction without an associated increase in CYP1A level (Fardel *et al.*, 1996). The cause of the discrepancy between *in vitro* and *in vivo* models is unknown but may be due to differences in metabolite formation between systems.

While our data also suggest that CYP1A and P-gp are not coordinately regulated, they may play complementary roles in cellular detoxification. P-glycoprotein may transport moderately hydrophobic CYP1A metabolites. Rat liver epithelial cells which were stably transfected with mouse CYP1A2 demonstrated induction of *mdr1* mRNA upon 2-acetylaminofluorene (2-AFF) treatment while parental cells lacking CYP1A2 showed no induction (Schrenk *et al.*, 1994). These data indicate that the CYP1A2 metabolite of 2-AAF may be responsible for elevated mdr1 mRNA in those rat hepatocyte cultures.

In addition to the liver, the gastrointestinal tract is an important site of cytochromes P450 activity. Induction of CYP1A in the gastrointestinal tract of freshly caught fish from all British Columbia field sites is probably due to dietary exposure to xenobiotics, as has been shown in previous studies of blennies exposed to inducers in the laboratory (Woodin et al., 1997). To determine if P-gp and CYP1A might be induced through dietary exposures at the Canadian sites, depurated blennies were fed isopods and amphipods exposed to sediment collected from Darrel Bay, a site within the pulp mill effluent stream. P-glycoprotein was not induced in liver of exposed fish relative to the levels in the controls, and was not detected in the gastrointestinal tract or any other organ. The same exposure did not induce CYP1A in the liver or gastrointestinal tract, suggesting that blennies were exposed to insufficient levels of inducers in the dietary and sedimentary material in this experiment to promote an increase in P-gp. To establish a relationship between exposure and P-gp expression level, a dose-response experiment in which groups of fish are exposed to different amounts of sediment and food from Darrel Bay could be undertaken.

Oil Exposure

Blennies exposed to oiled sediment exhibited significantly elevated hepatic P-gp levels compared to control fish. This increase in P-gp paralleled increases in CYP1A in these same blennies (Woodin et al., 1997). Surprisingly, blennies exposed only to oiled sediment had higher levels of both CYP1A and P-gp than did blennies both fed oiled food and maintained over oiled sediment. Although the difference is not statistically significant, this result might reflect a decreased feeding rate in animals exposed to high levels of petroleum. An inverse relationship between the fullness of gut and collection site contamination has been reported previously for the marine fish spot (Van Veld et al., 1990). However, whether this relationship is due to a decreased feeding rate or an increased food passage rate is unknown.

The observation that P-gp and CYP1A levels were significantly correlated in blennies exposed to oil is consistent with a previous study of P-gp and phase I enzyme activity in oil-treated fish (Kurelec, 1995). In that study, carp exposed to water contaminated with Diesel-2 oil at low levels (50 ppb PAH) showed no apparent induction of EROD in liver. However, the addition of the P-gp competitive inhibitor verapamil (2 µM) to the water containing oil stimulated a large induction of EROD activity in liver. Verapamil alone did not induce EROD. Presumably, verapamil inhibited P-gp-mediated efflux of some inducer, thereby increasing the internal dose in these fish to levels that had an effect equivalent to a 5-fold higher concentration of oil (Kurelec, 1995). The results with carp and our results suggest that some components of petroleum may be substrates for both P-gp and CYP1A.

The induction of CYP1A is most likely caused by large lipophilic multi-ring compounds in petroleum, such as benzanthracene, benzo[a]pyrene, and chrysene. P-glycoproteins transport structurally diverse compounds which nonetheless have the following general characteristics: substrates are moderately hydrophobic, amphipathic, low molecular weight, planar with a basic nitrogen atom, cationic or neutral but never anionic, and tend to be natural products (Gottesman and Pastan, 1988; Endicott and Ling, 1989; Pearce et al., 1990; Gottesman et al., 1994). Candidate P-gp inducers might include the heterocyclic aromatic nitrogen compounds found in petroleum, such as dibenzocarbazoles, quinoline, and aza-arenes, or CYP1A metabolites of PAHs. In addition, previous work suggests that benzo[a]pyrene exposure will induce P-gp in vitro (Fardel et al., 1996) but whether the inducer is the parent compound or a metabolite remains untested.

Whether the same compounds are acting to induce CYP1A and P-gp in these exposure experiments and whether the regulatory mechanism is similar for both proteins is not known. CYP1A1 is specifically induced by aryl hydrocarbon receptor agonists mainly via transcriptional regulation (Gonzalez et al., 1984) with some post-transcriptional control

(Pasco *et al.*, 1988). As indicated previously, it is possible that xenobiotic metabolites formed by CYP1A in hepatocytes may be acting to induce P-gp in the bile canaliculi. Elevated P-gp expression may occur via transcriptional (van der Heyden *et al.*, 1995) or by post transcriptional controls, including phosphorylation (Ratnasinghe *et al.*, 1998) and increased mRNA stability (Lee *et al.*, 1995).

Alternatively, P-gp induction may be part of a generalized defense mechanism against cell injury or DNA damage caused by cytotoxic xenobiotics (Chaundhary and Roninson, 1993; Kohno *et al.*, 1989) or physical stresses such as UV irradiation (Uchiumi *et al.*, 1993), heat shock or cadmium treatment (Chin *et al.*, 1990). Such cellular and genotoxic stresses are known to generate ceramides (Thévenod *et al.*, 2000), short chain lipids that stimulate the production of reactive oxygen species and initiate apoptosis (Green and Reed, 1998; Perry and Hannun, 1998), and activate the NF-κB transcription factor (Wiegmann *et al.*, 1994) which positively regulates mdr1 genes (Zhou and Kuo, 1997; Ogretmen and Safa, 1999). P-glycoproteins may play an anti-apoptotic role under conditions of physical stress by extruding ceramides (van Helvoort *et al.*, 1996; Thévenod *et al.*, 2000). In our study, we are not able to distinguish between P-gp induction due to direct interaction with xenobiotic substrates, CYP1A metabolites, or from indirect effects of cytotoxic xenobiotic non-substrates or physical stresses that might have affected the freshly caught fish from British Columbia field sites.

To fully elucidate interactions between CYP1A and P-gp, a better understanding of whether the parent compound or the CYP1A metabolite is a substrate or inducer for P-gp is necessary. P-glycoprotein and CYP1A may play complementary roles in cellular detoxification to protect organisms from accumulation of moderately hydrophobic xenobiotics or toxic endogenous metabolites. Future work on coexpression of P-gp and CYP1A in wild populations of fish may lead to novel insights about protective mechanisms

to avoid accumulation of natural product toxins and anthropogenic contaminants which may provide us with a greater understanding of human responses to toxins that individuals might be exposed to in, for example, the industrial workplace.

Conclusion

The average hepatic P-gp and CYP1A expression was elevated in blennies exposed to multiple xenobiotics in the field or to oil in the laboratory compared to control animals, but induction occurred in different cellular locations. What compounds are acting to induce P-gp and CYP1A in these exposure experiments is unknown. Not all exposed individual fish had elevated expression of both hepatic P-gp and CYP1A. BNF, the model CYP1A inducer and Ahr agonist did not induce P-gp in blennies. These results suggest that P-gp is not regulated by the aryl hydrocarbon receptor pathway, although P-gp and CYP1A may both be induced in blennies under certain exposure regimes. Induction of P-gps may be due to exposure to CYP1A parent compounds or metabolites, or to cellular stress from cytotoxic or genotoxic agents. While our data indicate that CYP1A and P-gp are not coordinately regulated, these proteins may play complementary roles in cellular detoxification. Thus P-gp activity may be an important mechanism of multixenobiotic resistance for organisms, such as intertidal fish, which are commonly exposed to environmental contaminants.

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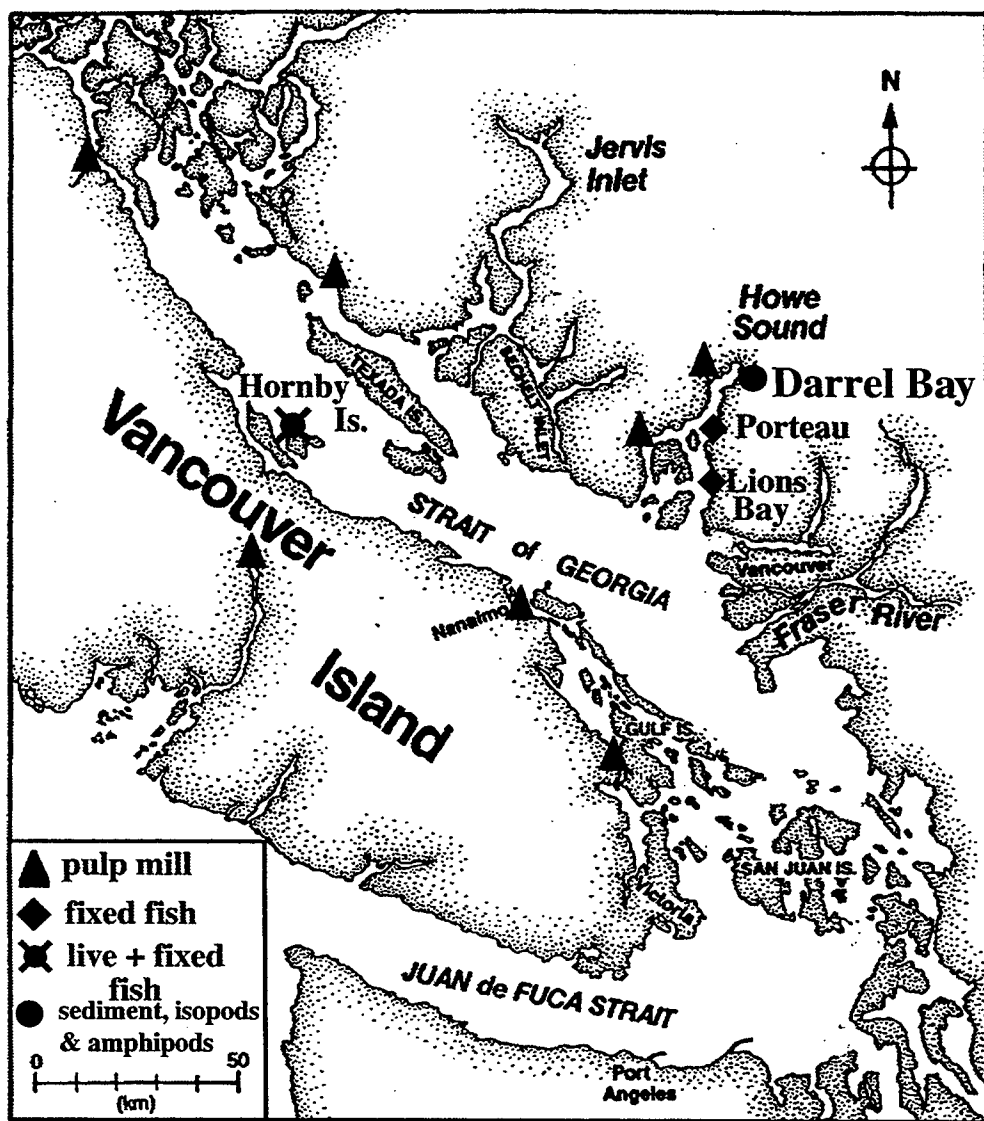
Figure 1. Location of pulp mills in the Strait of Georgia,
British Columbia, Canada. *A. purpurescens* were collected and
fixed in the field at Porteau Cove, Lions Bay, and Hornby
Island. Live fish were also collected from Hornby Island.
Sediment, wood fibres, isopods and amphipods were collected
from a pulp mill effluent exposed site at Darrel Bay.

A

~170 kDa

spleen
gill pool 2
gill pool 1
gonad pool 2
gonad pool 1
brain pool 2
brain pool 1
kidney pool 2
kidney pool 1
hind gut
fore gut
liver 2 (10 μg)
liver 1 (10 μg)
MW standard
liver 1 (50 μg)

B

~170 kDa

liver 5
liver 4
liver 3
liver 2
MW standard
liver 1
P-gp + control
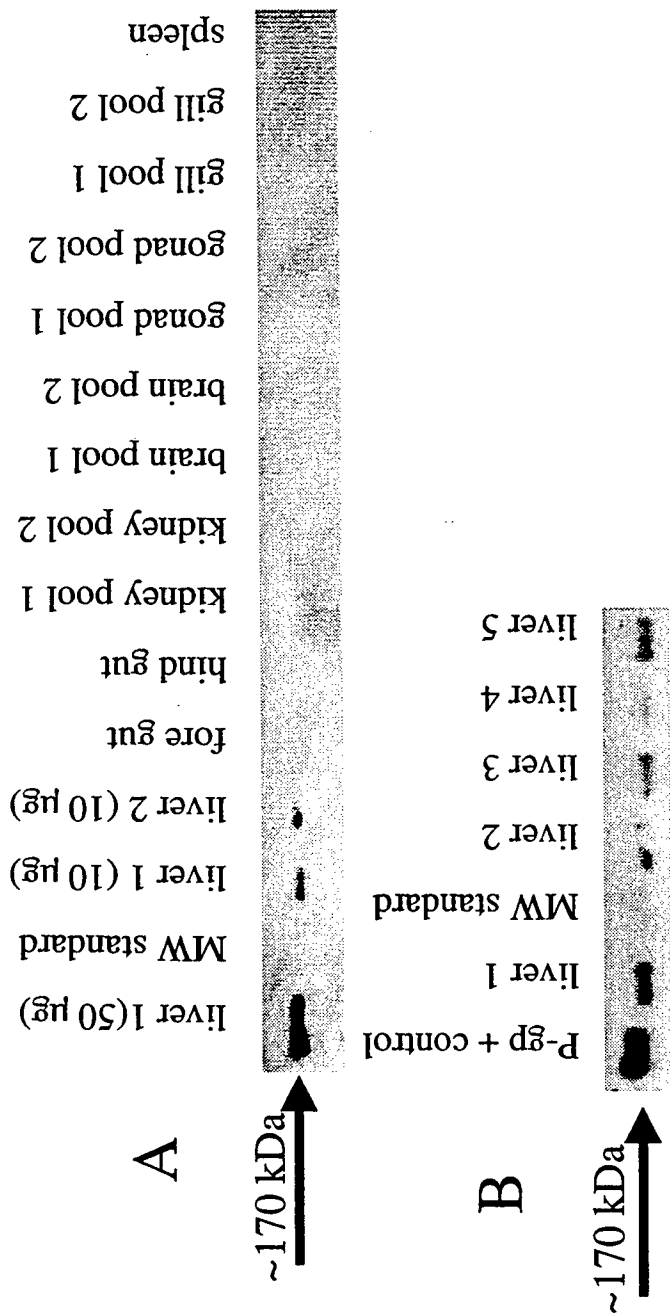
Figure 2. (A) Western blot of various organ lysates from *A. purpurescens* freshly collected from Lions Bay, Canada. Blots were probed with anti-P-glycoprotein mAb C219. 50 μg of tissue lysate samples were loaded for all nonhepatic organs. Liver lysates were loaded as labelled. (B) Interindividual hepatic P-glycoprotein variability is illustrated by *A. purpurescens* liver lysate samples (N=5) loaded at 5 μg. The P-glycoprotein positive (+) control is a 5 μg sample of killifish (*Fundulus heteroclitus*) liver.
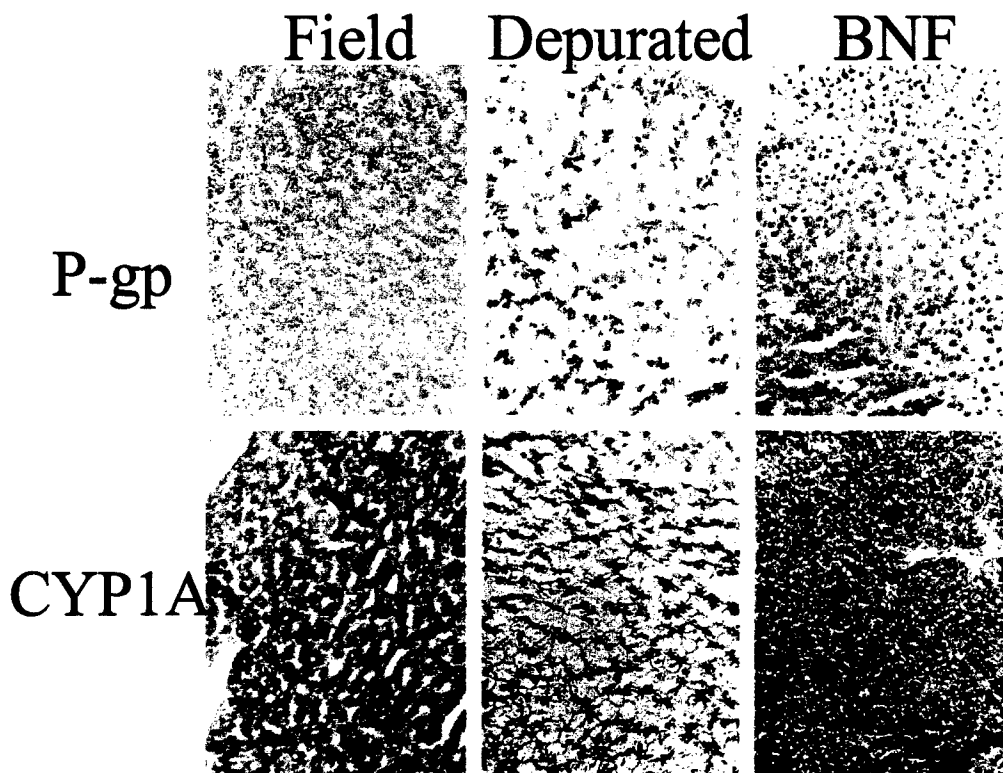
Figure 3. *A. purpurescens* liver immunohistochemical results. Red stain indicates immunoreaction. Top row (P-gp) is probed with anti-P-glycoprotein mAb C219. Bottom row (CYP1A) is probed with anti-cytochrome P450 1A mAb 1-12-3. Left column (Field): fish were freshly collected from Lions Bay, Canada. Note localization of P-gp stain to bile canaliculi and CYP1A stain to hepatocytes. Middle column (Depurated): fish were depurated in clean water in the laboratory for >1 month. No detectable stain for P-gp nor CYP1A. Right column (BNF): fish were depurated then exposed to ß-naphthaflavone by i.p. injection. No detectable stain for P-gp while hepatocytes stain for CYP1A.
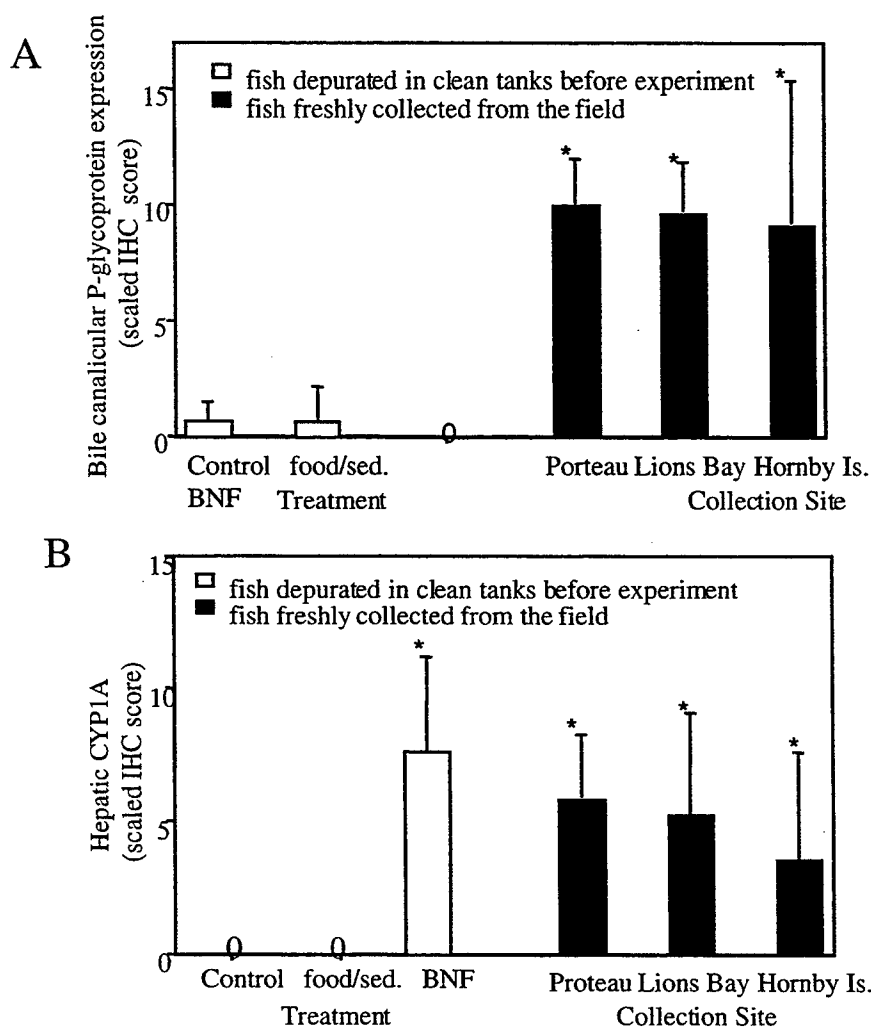
Figure 4. *A. purpurescens* were depurated in the laboratory for >4 week, then divided into three groups: control: i.p. injected with corn oil vehicle; food/sed.: exposed to food and sediment collected from a site within the pulp mill effluent stream; and BNF: i.p. injected with 10 μg ß-naphthoflavone/kg body weight. Fish were also freshly sampled from sites moderately exposed to pulp mill effluent (Porteau Cove and Lions Bay) and a site with low exposure (Hornby Island).

(A) Relative expression of P-glycoprotein in bile canaliculi. Sections were probed with anti-P-gp mAb C219. (B) Relative expression of cytochrome P450 1A in hepatocytes. Sections were probed with anti-CYP1A mAb 1-12-3. P-gp and CYP1A expression is reported as mean scaled immunohistochemical (IHC) staining score ± standard deviation as described in methods. (*) indicates significant difference from control (p<0.0001). (0) indicates either no P-gp or no CYP1A staining detected.
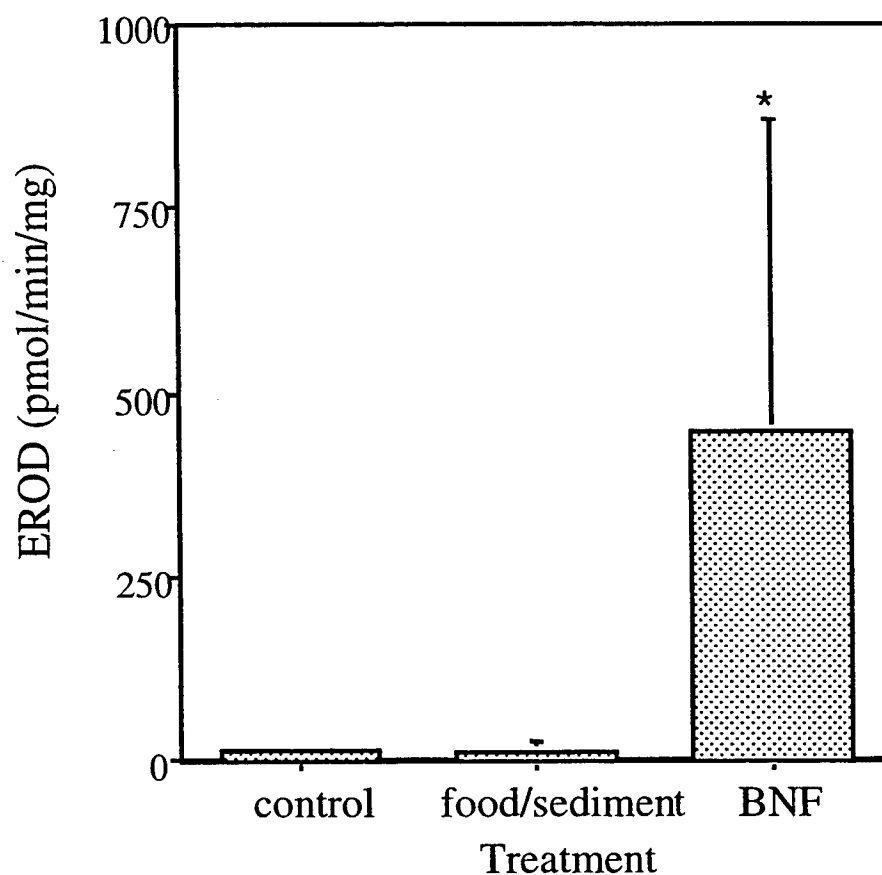
Figure 5. Ethoxyresorufin-O-deethylase (EROD) activity in hepatic microsomes from *A. purpurescens*. Fish were depurated in the laboratory for >4 week then divided into three groups: control: i.p. injected with corn oil vehicle; food/sed.: exposed to food and sediment collected from a site within the pulp mill effluent stream; and BNF: i.p. injected with 10 µg ß-naphthoflavone/kg body weight

Mean EROD activity ± standard deviations is reported. (*) indicates significant difference from control ($p < 0.001$).
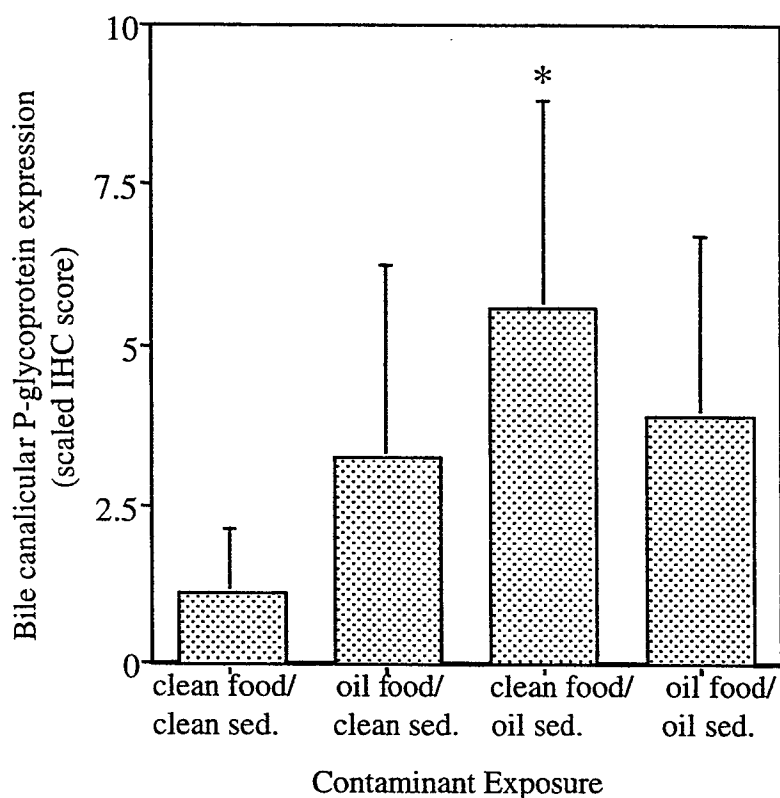
Figure 6. Relative expression of P-glycoprotein
in bile canaliculi of oil treated *A. purpurescens*.
Fish were collected from a reference site in
Alaska and depurated in clean tanks for 6
months. Fish (N=5) were then exposed to
combinations of either clean or oiled food and
sediment (sed.) Sections were probed with anti-
P-gp mAb C219. P-gp expression is reported as
the mean scaled immunohistochemical (IHC)
staining score ± standard deviation as described
in methods. (*) indicates significant difference
from cleanfood/clean sediment control ($p < 0.05$).
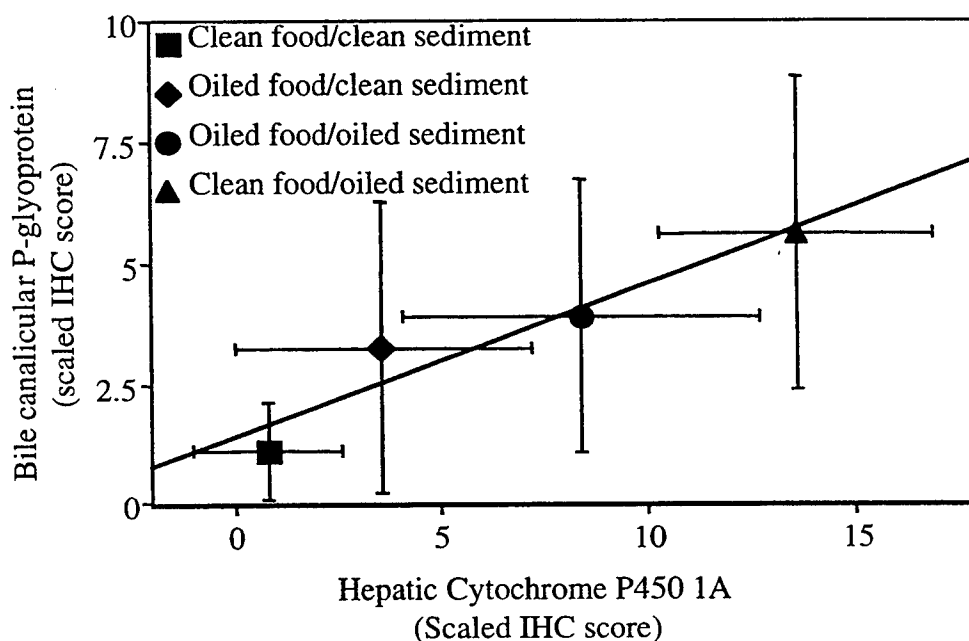
Figure 7. Correlation of bile canalicular P-glycoprotein expression versus hepatic cytochrome P450 1A expression in oil exposed *A. purpurescens* (y = 0.317x + 1.369 r^2 = 0.919). Fish were collected from a reference site in Alaska and depurated in clean tanks for 6 months. Fish (N=5) were then exposed to combinations of either clean or oiled food and sediment. Serial sections were probed with anti-P-gp mAb C219and anti-cytochrome P450 1A mAb 1-12-3. P-gp expression is reported as the mean scaled immunohistochemical (IHC) staining score ± standard deviation as described in methods. P-gp scores are taken from Figure 7 while CYP1A scores were previously reported by Woodin *et al.*, 1997.

Table 1. Cytochrome P450 1A expression in freshly collected and xenobiotic-exposed *A. purpurescens*

tissue/cell type	control	Food/Sed.	<u>Treatment/Field Collection Site</u> BNF	Porteau	Lions Bay	Hornby Is.
liver						
hepatocytes	0	0	7.6 ± 3.6^s	5.8 ± 2.5^s	5.2 ± 3.9^s	3.5 ± 4.1^s
vascular endothelium	0	0	10.0 ± 5.1^s	3.2 ± 2.2	2.8 ± 3.4	0.9 ± 2.0
bile duct	0	0	7.0 ± 1.4^s	3.5 ± 2.8^s	0.7 ± 1.2	0.5 ± 1.0
gill						
pillar cells	6.2 ± 0.4	4.0 ± 1.4	14.6 ± 1.0	0.4 ± 0.9	2.0 ± 2.8	2.3 ± 3.8
epithelium	6.2 ± 0.4	4.0 ± 1.4	14.6 ± 1.0	1.5 ± 1.7	4.0 ± 3.0	2.2 ± 3.9
vascular endothelium	6.2 ± 0.4	4.0 ± 1.4	14.6 ± 1.0	4.0 ± 2.5	4.6 ± 3.5	2.5 ± 3.7
kidney						
tubules	4.8 ± 1.5	4.0 ± 3.5	8.3 ± 4.7	8.5 ± 4.1	7.2 ± 4.2	7.2 ± 3.4
vascular endothelium	1.5 ± 1.9	0.7 ± 1.2	15.0 ± 0^s	2.0 ± 2.3	3.3 ± 4.7	1.8 ± 4.0
spleen						
parenchyma	1.0 ± 2.0	0	0	0	0	0
vascular endothelium	0.3 ± 0.6	0.3 ± 0.6	13.8 ± 2.7^s	0	0	1.2 ± 2.7
stomach						
epithelium	0	0	1.5 ± 3.4	1.6 ± 3.0		1.0 ± 1.7
vascular endothelium	0	0	14.1 ± 1.3^s	0	1.2 ± 2.7	0
compound gland	0	0	11.7 ± 1.7^s	2.4 ± 5.4	4.7 ± 6.0	1.8 ± 4.0
intestine						
epithelium	0	0	5.6 ± 4.7^s	3.7 ± 4.8	4.9 ± 3.2	2.6 ± 3.7
vascular endothelium	0	0	13.4 ± 2.1^s	0.7 ± 1.6	0.7 ± 1.6	0.4 ± 0.9
heart						
atrial endothelium	9.3 ± 1.2	8.3 ± 2.2	14.9 ± 0.3^s	9.8 ± 1.3	8.8 ± 3.2	8.6 ± 3.3
ventrical endothelium	9.3 ± 1.2	8.3 ± 2.2	14.9 ± 0.3^s	10.4 ± 2.1	9.6 ± 2.0	8.8 ± 3.0
gonad						
vascular endothelium	0	0	15.0 ± 0^s	0	0	0

Cellular localization of CYP1A induced in *A. purpurescens* freshly collected from the field
(Porteau Cove, Lions Bay, or Hornby Island) or depurated in the laboratory for more than one month
and then exposed to the model CYP1A induced BNF in corn oil, injected with the vehicle corn oil (control),
or exposed to food and sediment collected from Darrel Bay, a field site impacted by pulp mill effluent .
Values are reported as mean scaled immunohistochemical (IHC) staining score ± standard deviation as
described in Methods. (s) indicates significant difference (p<0.05) from control.

Chapter 5

Expression of P-glycoprotein in killifish *(Fundulus heteroclitus)* exposed to environmental xenobiotics.

Abstract

P-glycoproteins (P-gp) are transmembrane efflux flippases which prevent the cellular accumulation of moderately hydrophobic compounds and are responsible for certain multidrug resistance phenotypes in tumor cell lines and human patients. We investigated whether P-gps contribute to the xenobiotic resistant phenotype observed in a natural population of fish exposed to planar halogenated aromatic hydrocarbons (HAHs). Hepatic and intestinal epithelial P-gp expression was examined by immunoblot and immunohistochemistry in two populations of killifish *(Fundulus heteroclitus)*: a population from New Bedford Harbor (NB), MA, a Superfund site highly contaminated with planar HAHs; and a reference population from Scorton Creek (SC), Cape Cod, a relatively unpolluted site. The NB population has acquired resistance to the toxicity of planar HAHs. Hepatic P-gp levels were more than 40% greater in fish freshly collected from SC than in fish freshly collected from NB. When killifish from either site were maintained in clean water for up to 78 days to permit depuration of bioaccumulated contaminants, hepatic P-gp levels decreased approximately 50% by day 8. P-gp was detected in the intestinal epithelium in 55% of freshly collected NB fish. However, depurated NB fish and freshly caught and depurated SC fish rarely expressed P-gp in the intestine. In an effort to determine whether environmental chemicals at the two sites might contribute to altered P-gp expression, depurated fish were exposed to either sediment collected from SC or to a contaminant found at the NB site, 2,3,7,8-tetrachlorodibenzofuran (TCDF), a model aryl

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hydrocarbon receptor (AhR) agonist. Neither exposures affected hepatic P-gp levels in killifish. Elevated intestinal P-gp in NB fish might provide a barrier against absorption of P-gp substrates/inducers and thus limit the amount of these compounds reaching the liver which might account for the lower hepatic P-gp levels in NB fish compared to SC fish. The differences in elevated hepatic P-gp levels (SC>NB) and intestinal P-gp (NB>SC) in freshly collected fish might also reflect differing environmental exposure to anthropogenic contaminants or microbial, algal, plant or other natural products via the water column, sediment, or diet at each site.

Keywords: P-glycoprotein; multidrug resistance; multixenobiotic resistance; fish; aquatic environment; 2,3,7,8-tetrachlorodibenzofuran.

Introduction

P-glycoproteins (P-gps) are responsible for certain multidrug resistance (MDR) phenotypes in tumor cell lines (Juliano and Ling, 1976) and in human patients (Gerlach *et al.*, 1986.) We investigated whether P-gps contribute to the xenobiotic resistant phenotype observed in a natural population of fish exposed to planar halogenated aromatic hydrocarbons (HAHs). P-glycoproteins function as energy dependent efflux flippases that prevent the cellular accumulation of a wide variety of moderately hydrophobic compounds. Known P-gp substrates include endogenous regulators such as glucocorticoids (Naito *et al.*, 1989; Ueda *et al.*, 1992), drugs and other natural products (Gottesman and Pastan, 1988), and environmental contaminants (Phang *et al.*, 1993; Cornwall *et al.*, 1995; Bain and LeBlanc, 1996). P-glycoproteins can be induced by exposure to compounds of both natural and anthropogenic origin (Gant *et al.*, 1991; Fardel *et al.*, 1996; Schrenk *et al.*, 1994).

In the present study, our objectives were to compare the levels of P-gp expression in multiple organs in natural populations of vertebrates, killifish *Fundulus heteroclitus*, collected from a contaminated site (New Bedford Harbor (NB, MA)) and from a relatively unpolluted reference site (Scorton Creek (SC) on Cape Cod, MA). New Bedford Harbor is a Superfund site in southeastern Massachusetts which is highly contaminated with polychlorinated biphenyls (PCBs), heavy metals and planar halogenated aromatic hydrocarbons (Ah), such as dibenzofurans, as well as containing lower quantities of dibenzo-p-dioxins (Pruell *et al.*, 1990). Killifish were selected as the model study organism because they are non-migratory and have small home ranges (Lotrich, 1975), and thus reflect exposure at the site where they are collected. Killifish at NB have a high body burden of total PCBs (272 μg ΣPCB/g dry tissue) compared to killifish collected from SC (0.177 μg ΣPCB/g dry tissue) (Bello, 1999). The high level of pollutants at NB has resulted in killifish there acquiring resistance to the toxic effects of planar HAHs. Two P-gp genes have been described in *F. heteroclitus* (Cooper, 1996), and furthermore, elevated expression of hepatic P-gps in a polycyclic aromatic hydrocarbon-resistant population of killifish (Cooper *et al.*, 1999) have been reported. Thus we hypothesize that NB fish would show elevated P-gp expression, which could contribute to the resistant phenotype in this population.

We examined P-gp expression in freshly collected fish from SC and NB and in fish that were collected from these sites and then maintained in clean water for up to 78 days, a regimen which should permit depuration of contaminants. We detected P-gp expression only in liver and intestine, the predominant organs involved in interactions with chemicals from the environment. Differences in hepatic P-gp levels (SC>NB) and in intestinal P-gp (NB>SC) in freshly collected fish might reflect differing environmental exposure to anthropogenic contaminants or microbial, algal, plant or other natural products via the water column, sediment, or diet at each site. In an effort to determine whether

environmental chemicals at the two sites might contribute to altered P-gp expression, we exposed depurated fish to either sediment collected from SC or to a contaminant found at the NB site, 2,3,7,8-tetrachlorodibenzofuran (TCDF), a model aryl hydrocarbon receptor (AhR) agonist (Pruell et al., 1990). A structurally related AhR agonist, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), has been suggested to induce P-gp in rat liver (Burt and Thorgeirsson, 1988). Neither sediment exposures nor 2,3,7,8-tetrachlorodibenzofuran treatment affected P-gp levels in killifish. Our results suggest that P-gp induction may play a role in resistance against moderately hydrophobic compounds at both NB and SC but might not be involved in resistance to highly lipophilic contaminants such as planar HAHs. The evaluation of P-gp expression in natural populations, will help us to better understand the biochemical mechanisms employed to permit animals to survive in environments containing both naturally occurring toxins and anthropogenic contaminants.

Methods

Specimen Collection and Maintenance

For the depuration study, *Fundulus heteroclitus* were collected by fish trap and cast net from the reference site Scorton Creek, MA on September 14, 1998. Scorton Creek was selected as a reference site because killifish collected from this site in 1994 have been shown to have low body burden of 45 tested PCB congeners (0.177 µg ΣPCB/g dry tissue) and analysis of killifish collected in 1997 showed low levels in liver (1.11 µg ΣPCB/g dry tissue) (Bello, 1999). Fish were maintained in 20° C free flowing seawater from Vineyard Sound in a single tank at the Environmental System Laboratory of Woods Hole Oceanographic Institution. Killifish were also collected by fish trap from the PCB contaminated site New Bedford Harbor, MA by the EPA/Narragansett team on September 24, 1998. Killifish collected from New Bedford in 1994 have been shown to have high

body burden of total PCBs (272 µg ΣPCB/g dry tissue) and analysis of killifish collected in 1997 showed high levels in the liver (809 µg ΣPCB/g dry tissue) (Bello, 1999). Fish were held in 20° C seawater from Narragansett Bay at the EPA/Narragansett lab for one week. Fish were then transported to Woods Hole where they were maintained in a single tank as described above.

Killifish used in the sediment exposure experiment were collected from Scorton Creek during July 1999 and were maintained in the laboratory for 4 months as described above, before the onset of the experiment (see below). Killifish used in the TCDF dosing experiment were collected from New Bedford during July 1995 and from Scorton Creek during August, 1995. These fish were maintained in the laboratory for approximately 17 months before TCDF exposures (see below).

Depuration experiment

Killifish from Scorton Creek and New Bedford were maintained in clean sea water for up to 78 days to permit depuration of bioaccumulated contaminants. A subsample of fish were sacrificed after 1, 8, 22, 44, and 78 days post-collection from the field. For each time point we subsampled 15 males and 15 females from Scorton Creek plus 10 males and 10 females from New Bedford Harbor.

Fish were sacrificed by cervical scission. Kidney, gill, gonad, gastrointestinal tract, brain, spleen, heart, and small portions of liver were fixed in 10% neutral buffered formalin and embedded in paraffin cassettes for immunohistochemical analysis. Before embedding, gills were decalcified by a 24 hour immersion in a solution of 10% (w/v) sodium citrate and 25% formic acid. The remaining portion of liver from each fish was snap frozen in liquid nitrogen and stored at -70° C until Western blot analysis was completed.

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Sediment Exposure

Approximately 25 litres of surface sediment were collected from Scorton Creek on October 6, 1999. Sediment was stored at 4° C in the dark for 2 weeks before the experiment began. Three tanks were lined with 3 cm of sediment and covered with 15 cm of standing seawater. Air stones were used to aerate the standing water. An additional three tanks containing no sediment were filled with 15 cm of standing water. On October 20, 1999, 45 female fish were randomly selected from a tank with clean water containing fish originally collected four months previously from Scorton Creek (June, 1999). Fifteen of these depurated fish that had been held in clean water for >4 months were randomly selected as controls and were sacrificed on the first day of the experiment. To control for tank effect, 5 fish were randomly assigned to each of 3 sediment lined tanks and 3 standing water tanks (15 fish total per treatment). Fish were maintained in sediment or control tanks for 8 days before fish were sacrificed by cervical scission and livers were dissected. Livers were snap frozen in liquid nitrogen and stored at -70° C until Western blot analysis was completed.

TCDF exposure

In our study, we examined archived samples which had been previously obtained (Bello, 1999). In the original study, male killifish from Scorton Creek and New Bedford, which had been maintained in the laboratory in clean water for approximately 17 months, received intraperitoneal (i.p.) injections of 7.6 nmol TCDF/kg body weight (Sigma, St. Louis, MO) or a corn oil vehicle control. Nine fish from each site were injected with TCDF and another 9 from each site with corn oil. To control for tank effect 12 tanks were used during the experiment, the 9 fish from each treatment/site group were randomly assigned to 3 tanks such that there were 3 fish per tank and 3 tanks per treatment for each site. One additional tank contained 3 fish originally from New Bedford which had been

injected with 50 nmol TCDF/kg body weight. Three days post-injection, fish were sacrificed. Livers from all fish and extrahepatic tissues (heart, gill, intestine, kidney, spleen, and testes) from one fish in each tank (N=3 for each treatment except N=1 for 50 nmol/kg group) were fixed in 10% neutral buffered formalin and embedded in paraffin for immunohistochemical analysis

Western Blot for P-glycoprotein

Processing of liver and subsequent immunoblot analysis for P-gp expression followed the protocols previously described (Cooper *et al.*, 1996) using the affinity purified murine monoclonal antibody (mAb) C219 (IgG$_{2a}$) (Signet Laboratories, Inc., Dedham, MA) which is immunoreactive against mammalian P-gps (Kartner *et al.*, 1985). C219 recognizes an internal, highly conserved amino acid sequence common to MDR1 and MDR2(3) of the 170 kDa transmembrane glycoprotein and all other MDR isoforms whose sequence is known (Kartner *et al.*, 1985; Endicott and Ling, 1989; Georges *et al.*, 1990). A comparison of the mAb C219 epitope with the known partial sequence of the two P-gp isoforms previously identified in killifish (Cooper, 1996) suggests that mAb C219 should cross react with both isoforms in killifish and a previous study showed that a single band at approximately 170 kDa is detected for liver lysates (Cooper *et al.*, 1996).

The protein concentrations of tissue lysates were determined using the bicinchoninic acid (BCA) procedure (Smith *et al.*, 1985). Tissue lysates were diluted to 5 µg total protein with standard SDS-PAGE sample buffer, denatured (65° C, 4 min), and loaded onto a 4-12% tris glycine gel (Novex, San Diego, CA). Pooled mummichog liver lysates (*Fundulus heteroclitus*) at 2.5 µg, 5.0 µg, 7.5 µg, and 10 µg were loaded on each gel as controls for between run variability. Electrophoresis and electrotransfer to nitrocellulose membranes followed the manufacturer's recommendations (Novex, San Diego, CA).

Transfer efficiency and protein loading consistency was checked by Ponceau staining the nitrocellulose. Membranes were blocked in 5% (w/v) non-fat dry milk in TTBS (100 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.02% Tween 20) and incubated for 1 hour in mAb C219 (Signet, Dedham, MA) (2.5 µg/ml in TTBS with 1% (w/v) non-fat dry milk). The membrane was washed 3 times with TTBS and incubated with secondary antibody (SAM-HRP sheep anti-mouse IgG horseradish peroxidase (diluted 1:1000 with TBS, 5% (w/v) non-fat dry milk) (Amersham Life Sciences, Little Chalfont, England). The blot was incubated with the ECL reagents luminol, p-coumerin, and hydrogen peroxide, and then pressed against autoRad X-ray film as previously described (Matthews *et al.*, 1985).

Developed film was photographed with a digital camera and integrated densitometry measurements were taken using the NIH Image computer program in order to estimate relative P-gp content in liver extracts. For the depuration experiment, P-glycoprotein content in each sample was expressed in arbitrary units made by assigning a value of 100 to the signal obtained for 5 µg of the pooled *F. heteroclitus* liver lysate. For the sediment exposure experiment, P-glycoprotein content in each sample was expressed in arbitrary units made by assigning a value of 10 to the signal obtained for 10 µg of a different pooled *F. heteroclitus* liver lysate. Values for arbitrary units cannot be compared between depuration and sediment experiments.

Immunohistochemical analysis

P-glycoprotein detection

Paraffin-embedded samples were sectioned at 5 µm and mounted on poly-L-lysine-coated slides (Sigma, St. Louis, MO), deparaffined, and then hydrated with 0.5% bovine serum albumin/0.5% nonfat dry milk in TPBS (0.05% Tween 20 in PBS) and immunochemically stained using an indirect peroxidase stain (Universal Immunoperoxidase

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Staining Kit (Murine), Signet Laboratories, Inc., Dedham, MA) with mAb C219 served as the primary antibody (10 μg/ml in TPBS) as previously described (Hemmer *et al.*, 1995).

Stain quantification

Specific staining by mAb C219 was evaluated by light microscopic examination of the stained sections. Cell types that stained and their associated occurrence and staining intensity were recorded for each tissue section examined. Staining results were recorded as intensity in any one cell type compared to reference specimens previously designated as staining with either high or low intensity: negative (0), mild (1), mild/moderate (2), moderate (3), strong (4) or very strong (5); and as degree of occurrence in any one cell type: absent (0), rare (1), some (1.5), many (2) and all (3). Quantitative comparisons were made between tissue types at various sites by using the product of intensity and occurrence, giving a range of score from 0 to 15. This scoring scheme has been described previously (Woodin *et al.*, 1997).

Statistical Analyses

Differences in mean P-gp content between day 1 and depurated groups, and between field site populations were statistically analyzed by one-way ANOVA using as appropriate Fisher's Protected LSD or Dunnett's one-tailed procedure for unequal sample sizes using the SuperANOVA (Abacus Concepts) statistical program. To analyze the effect of depuration and field site together, a two-way ANOVA using the StatView statistical program was employed. $P \leq 0.05$ was accepted as significant.

Results

P-glycoprotein expression in depurated *Fundulus heteroclitus*

Hepatic P-gp content was quantified by Western blot analysis of liver lysates. A single somewhat diffuse band at approximately 170 kDa was immunoreactive with mAb C219 (Figure 1). Monoclonal antibody C219 recognizes an epitope common to both known killifish P-gps and thus we cannot distinguish between expression of isoform fpgpA responsible for bile acid export (Ballatori *et al.*, 2000) from isoform fpgpB, the presumed multixenobiotic resistance protein (Cooper, 1996). There was large interindividual variability in hepatic P-gp content observed in killifish from both sites. This required the use of large numbers of animals in these experiments for statistically significant results to be generated (N=30 for SC and N=20 for NB). Hepatic P-gp expression was significantly greater (41% greater) in freshly collected fish sampled from the reference site SC than freshly collected fish sampled from the contaminated site NB (Figure 2). Fish maintained in clean water showed a decrease in hepatic P-gp expression which was apparent as early as 8 days post-collection in fish from both sites (Figure 2). The average hepatic P-gp level in fish from SC depurated for 8 or more days was 45% lower than the average level in freshly collected fish from SC. In the same comparison for NB, the average hepatic P-gp for depurated fish was 54% lower than for freshly caught fish. Average levels of hepatic P-gp expression among all depurated fish from SC was 68% greater than that in depurated NB fish. No difference in hepatic P-gp expression was observed between female and male fish from either site or at any sampling time during depuration.

The cellular localization of P-gp was examined in paraffin embedded fixed tissue sections probed with mAb C219. P-glycoprotein expression was localized to the bile canaliculi in liver and to epithelial cells in the posterior portion of the gastrointestinal tract (Figure 3). P-glycoprotein was not detected by immunohistochemistry in other organs

examined, including kidney, gill, gonad, brain, spleen, and heart. Fifty-five percent of killifish from NB sampled 1 day after collection had detectable expression of P-gp by immunohistochemistry in the hind gut (Figure 4). Intestinal P-gp expression was also detected in one fish sampled 8 days after collection from NB but no other fish from NB sampled up to 78 days post-collection expressed P-gp in intestine. Among fish collected from SC, few individuals (1 or 2, per 30) expressed P-gp in the intestinal epithelium on post-collection days 1, 8, and 22. No fish from SC had detectable levels of intestinal P-gp expression 44 or 78 days after collection. No difference in prevalence of P-gp expression in the intestinal epithelium was observed between female and male fish.

Effect of sediment exposure on hepatic P-glycoprotein expression

In an effort to determine whether environmental chemicals at the SC site might be responsible for the elevated levels of P-gp observed in livers of freshly collected SC fish, we tested whether exposure to sediment collected from SC could cause an increase in hepatic P-gp expression. Female fish from SC which had been maintained in clean water for >4 months were exposed to SC sediment for 14 days. As measured by Western blot, this sediment exposure regime did not stimulate an elevation of hepatic P-gp in these fish (Figure 5).

Effect of TCDF exposure on P-glycoprotein expression

To determine if environmental exposure to highly lipophilic compounds such as planar HAHs might be responsible for the elevated hepatic and intestinal P-gp levels observed in freshly collected fish from NB, we tested whether exposure to a model AhR agonist known to be present in NB sediment, TCDF, might have effects on P-gp expression in depurated fish collected from either SC or NB. In all treatment groups, immunohistochemical examination revealed P-gp only in the liver, where it was localized to

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the bile canaliculi. P-glycoprotein expression was not detected in any of the other organs examined including the gastrointestinal tract, heart, gill, kidney, spleen, and testes. No significant difference in hepatic P-gp expression was observed between depurated killifish from either NB or SC which were treated with either TCDF or the vehicle control (Figure 6).

Discussion

Hepatic P-gp levels were more than 40% greater in freshly collected SC fish than in NB fish. In contrast, P-gp was detected in the intestinal epithelium in 55% of freshly collected NB fish while few freshly caught SC fish expressed P-gp in this organ. The mAb C219 that was used in Western blots and immunohistochemistry to visualize and quantify P-gp expression, recognizes an epitope common to both known killifish P-gps (Kartner et al., 1985; Cooper, 1996). Due to the unavailability of specific probes, we are unable to distinguish between expression of isoform fpgpA responsible for bile acid export (Ballatori et al., 2000) from isoform fpgpB the presumed multixenobiotic resistance protein (Cooper, 1996). Spgp/BSEP, the mammalian homolog for fpgpA, has been detected not only in the liver but also in brain and in mucosa of small and large intestine (Török et al., 1999). A broader tissue distribution has been reported for the mammalian xenobiotic P-gp transporter, which is found at high levels in liver, kidney, small and large intestine, blood-testes barrier, blood-brain barrier, and adrenal cortex (Fojo et al., 1987; Thiebaut et al., 1987; Sugawara et al., 1988; Cordon-Cardo et al., 1990; Sugawara, 1990). We do not know if the elevated expression of P-gp(s) found in the liver of freshly caught SC fish is due to the same isoform(s) as that responsible for the elevated P-gp(s) levels in posterior intestine of freshly caught NB fish.

When killifish from NB and SC were maintained in clean water, hepatic P-gp levels decreased approximately 50% in fish from each site within 8 days. Fish were maintained

in the laboratory for up to 78 days to permit depuration of the highly lipophilic contaminants such as PCBs which have bioaccumulated to elevated levels in the NB fish (272 µg/g dry tissue) (Bello, 1999). Other researchers have shown that ΣPCBs are eliminated from live fish with a half-life of approximately 4.5 months (Elskus et al., 1999; Lake et al., 1995). Thus despite the long period of depuration that the NB fish underwent, after 78 days the fish could be expected to retain approximately 182 ΣPCB µg/g dry tissue, 3 orders of magnitude higher than the ΣPCB concentration in killifish from the uncontaminated site at Scorton Creek (<0.2 ug/g) (Bello, 1999; Elskus et al., 1999). That hepatic P-gp levels decreased by day 8 and then did not decrease further at later sampling points suggests that rather than highly lipophilic compounds, moderately hydrophobic compounds, which are likely to be more rapidly mobilized and depurated from the fish, could possibly be responsible for the elevated P-gp levels in liver and intestine observed in freshly caught fish.

The elevation in hepatic P-gp levels in freshly caught fish from both sites compared to fish maintained in the lab was not as pronounced as the difference in P-gp levels between freshly caught and depurated fish we previously observed in another fish species, the high cockscomb blenny (Anoplarchus purpurescens) (Bard et al., 2000 in preparation). Freshly collected blennies had a >14 fold increase in hepatic P-gp levels compared to blennies maintained in clean water in the laboratory (Bard et al., 2000 in preparation). Furthermore, the elevation of expression we observed in freshly caught killifish is relatively small compared to increases in P-gp resulting from in vivo drug exposure in mammals: rats exposed to 2-acetylaminofluorene or 3-methylcholanthrene for 36 hours showed a 5-fold increase in hepatic P-gp levels (Gant et al., 1991); monkeys had up to 15-fold, 13-fold, and 6.5-fold increases in hepatic P-gp expression upon a 7 day exposure to either erythromycin, rifampicin, or tamoxifen respectively (Gant et al., 1995). One possible explanation for the limited decrease in hepatic P-gp levels we observed in killifish may be

that the laboratory food may contain inducers for hepatic P-gp isoform(s). Unknown to us at the time, the fish flake food Tetramin that we used has been shown to stimulate elevated hepatic P-gp in killifish which were collected from unpolluted field sites (personal communication K. Karnacky, 2000). The average hepatic P-gp levels in depurated SC fish were approximately 50% greater than those in depurated NB fish. Several possibilities may explain why this trend persisted during the depuration period in which the two populations of fish were in identical environments and fed the same diet: the threshold for induction of hepatic P-gp isoform(s) may be greater in NB fish than SC fish; the SC population may have a genetic predisposition for elevated constitutive levels of hepatic P-gp compared to the NB population; lower hepatic P-gp in NB fish might be due to lower inductive capability due to some undefined negative impact associated with the high body burden of contaminants; or alternatively, NB fish might have preferentially invested in other hepatic resistance mechanisms, such as induction of suites of detoxification proteins or other classes of transporters, thus making the rapid induction of high levels of hepatic P-gps prohibitively costly for the fish.

The order of magnitude elevation of intestinal P-gp levels in freshly collected NB fish (2.3 ± 2.8 scaled IHC score) compared to NB fish depurated for 8 days (0.1 ± 0.7 scaled IHC score) and compared to all fish from SC (0.2 ± 1.1 scaled IHC score), is slightly larger than increases in P-gp resulting from *in vivo* drug exposure in mammals: in human patients rifampin treatment increased intestinal P-gp content 3.5-fold (Greiner *et al.*, 1999) while in rats cisplatin increased intestinal P-gp expression by >2 fold (Demeule *et al.*, 1999) and dexamethasone treatment in rats elevated intestinal P-gp levels by >2 fold (Lin *et al.*, 1999). Elevated P-gp levels in the intestine of NB fish suggests that P-gp inducers may be present in the diet of these fish. That few depurated fish from either site showed any expression of P-gp in the intestine suggests that the Tetramin fish food does not contain high levels of inducers for the intestinal P-gp isoform(s).

In an effort to determine whether environmental chemicals at each site might be responsible for elevated P-gp levels in freshly collected fish, we examined the inducing potential of first, sediment collected from SC, and second, TCDF a model AhR agonist present at high levels at the NB site. No change in hepatic P-gp expression was detected is depurated SC fish exposed to SC sediment. However, visual observations of the behaviour of the killifish and condition of the sediment showed that fish swam in the open water but did not burrow into the sediment or associate with it in any way during this experiment. The storage of the sediment in the dark and cold for 2 weeks prior to the onset of the experiment might have disrupted the production of natural products by bacterial, fungal, and plant growth previously established in the sediment while in the estuary environment. Natural products produced by bacteria isolated from estuary sediment have been shown previously to interact with P-gp and possibly be substrates and/or inhibitors of the transporter (Toomey *et al.*, 1996). Such natural products if bound to the surface sediment could be released into the water stream, when the sediment is disturbed by tidal action or animal foraging. Thus we suggest that our experiment did not adequately replicate the sedimentary exposure that fish experience in the wild. Our results do not exclude the possibility that unknown components in the environment at Scorton Creek, including natural products in the sediment, might be responsible for the elevated level of P-gp observed in the liver of fish collected from this site.

In the second experiment, depurated killifish originally collected from both SC and NB sites were exposed to TCDF. There were no changes in hepatic or intestinal P-gp levels upon exposure to doses as high as 50 nmol TCDF/kg body weight. Early studies of TCDD-treated rats reported coinduction of hepatic *mdr* and CYP1A2 mRNA (CYP1A1 was not examined) suggesting an AhR-mediated induction of P-gp (Burt and Thorgeirsson, 1988). Subsequent work has not supported this claim: similar exposures in mice have not detected increased P-gp levels and the investigators concluded that mdr gene induction does

not occur via the AhR pathway (Teeter *et al.*, 1991). The lack of hepatic P-gp increase in TCDF-treated fish also suggests that TCDF may not yield a metabolite that is a P-gp substrate or inducer. Exposures of blennies and killifish respectively to the aryl hydrocarbon receptor agonists β-naphthoflavone (Bard *et al.*, 2000 in preparation) and 3-methylcholanthrene (Cooper *et al.*, 1999) also have not stimulated elevated levels of hepatic P-gp. Together, these results strongly suggest that in teleost fish, P-gp induction in unlikely to occur via the aryl hydrocarbon receptor pathway.

What compounds in the environment may be directly responsible for the elevated P-gp expression observed in freshly collected fish in unknown. Possibly, the elevated P-gp in NB fish may be part of a generalized defense mechanism against cell injury or DNA damage caused by cytotoxic xenobiotics (Chaundhary and Roninson, 1993; Kohno *et al.*, 1989) or physical stresses such as UV irradiation (Uchiumi *et al.*, 1993), heat shock or cadmium treatment (Chin *et al.*, 1990). Such cellular and genotoxic stresses are known to generate ceramides (Thévenod *et al.*, 2000), short chain lipids that stimulate the production of reactive oxygen species and initiate apoptosis (Green and Reed, 1998; Perry and Hannun, 1998), and activate the NF-κB transcription factor (Wiegmann *et al.*, 1994) which positively regulates mdr1 genes (Zhou and Kuo, 1997; Ogretmen and Safa, 1999). However, histological examination does show cellular pathology associated with an increased prevalence of parasitic infection in NB fish (Bello, 1999). Thus we are not able to distinguish between P-gp induction due to direct interaction with xenobiotic substrates or from indirect effects of cytotoxic xenobiotic non-substrates or physical stresses that might have affected the freshly caught fish from SC and NB field sites.

We had anticipated that if P-gp-mediated transport of xenobiotics contributes to the resistant phenotype observed in NB fish, then elevated P-gp expression would be detected in liver and intestine of these fish compared to SC fish. However in contrast to expectations, hepatic P-gp levels were lower in NB fish than SC fish. Consistent with our

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expectations, P-gp expression was detected in the intestinal epithelium of the majority of freshly collected NB fish compared to few SC fish. Elevated expression in different organs might be due to tissue specific expression of the two fish P-gp isoforms, which we were unable to distinguish between in our experiment. If the two fish P-gp isoforms follow the trend seen in mammalian P-gp isoforms, we might expect that these transporters might have different substrate specificities and be induced by exposure to different compounds but also display some substrate/inducer overlap. The elevation of intestinal P-gp levels observed only in freshly collected NB fish suggests that inducers for the gut specific isoform might not be present above the threshold for induction either at SC or in the laboratory environment. Dietary exposure to inducers at NB is probably responsible for elevated intestinal P-gp isoform levels. The route of exposure for the liver may be via the diet or through diffusion of inducers from the water column or sediment to the skin or gills. If an inducer were to enter the body only via the later routes then elevated hepatic P-gp would not be accompanied by elevated intestinal P-gp, the trend observed in SC fish.

One hypothesis to explain the trend observed in NB fish is that intestinal P-gp isoform in NB fish might be induced only by dietary exposure to compounds present at NB but due to overlapping specificity may also transport some substrate/inducers which interact with the liver P-gp isoform(s). In this way, elevated intestinal P-gp may provide a barrier against absorption of hepatic P-gp substrates/inducers and thus limit the amount of these compounds reaching the liver which might account for the lower hepatic P-gp isoform(s) levels in NB fish compared to SC fish. If this interpretation of the results is correct, then P-gp(s) may play a role in the resistance this population has acquired to the toxic effects of some classes of contaminants at the NB site.

The lack of induction of P-gp in TCDF-treated fish and the fact that hepatic and intestinal P-gp levels did not further decrease after 8 days depuration, suggest that moderately hydrophobic compounds rather than highly lipophilic contaminants, such as

planar HAHs, might be responsible for P-gp induction. The basis for differences in elevated levels of hepatic P-gp isoform(s) (SC>NB) and intestinal P-gp isoform(s) (NB>SC) in freshly collected fish might reflect differing environmental exposure to moderately hydrophobic anthropogenic contaminants or microbial, algal, plant or other natural products via the water column, sediment, or diet at each site.

Acknowledgements

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201 kD →

122 kD →
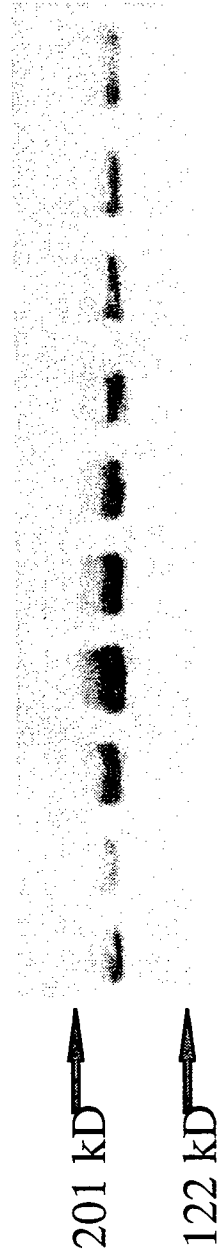
Figure 1. Interindividual hepatic P-glycoprotein variability is illustrated by Western blots of liver lysates from *F. heteroclitus* sampled one day after fish were collected from the reference site at Scorton Creek, MA. Blot was probed with anti-P-glycoprotein mAb C219 which reacted with a band at approximately 170 kD. 5 μg of liver lysate samples (N=10) were loaded in each lane.
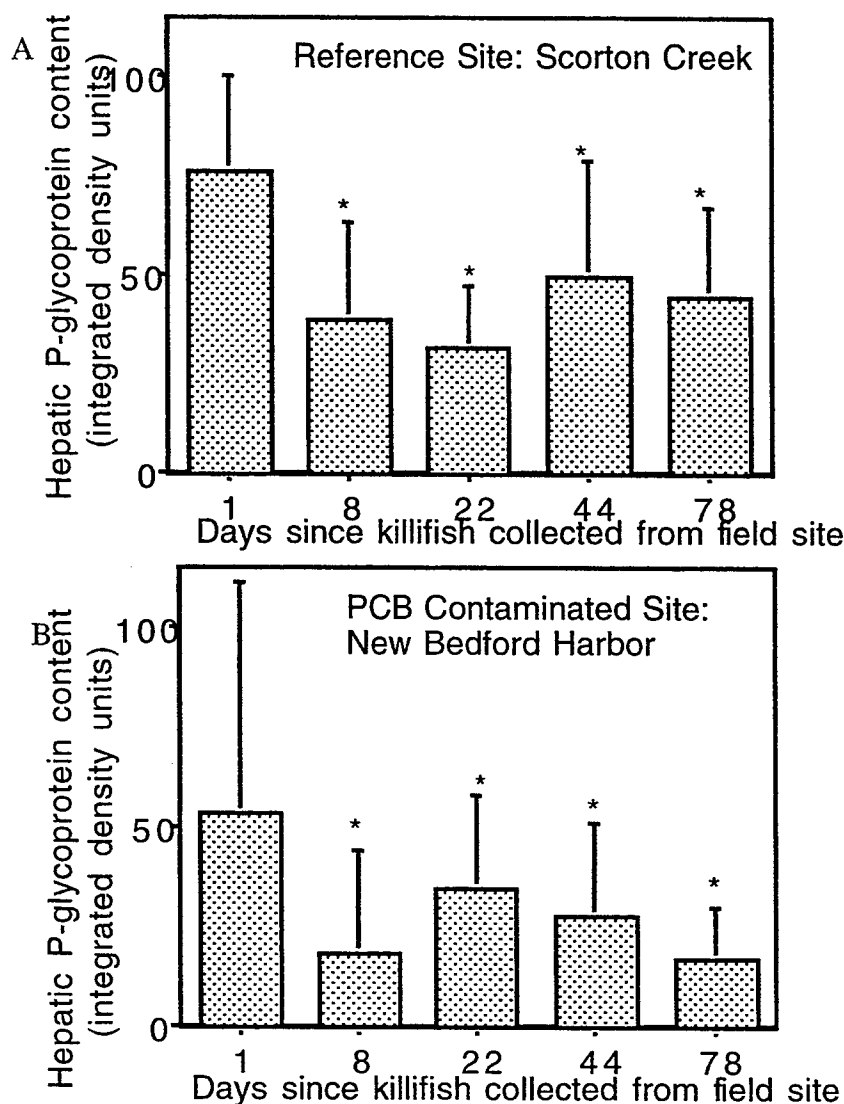
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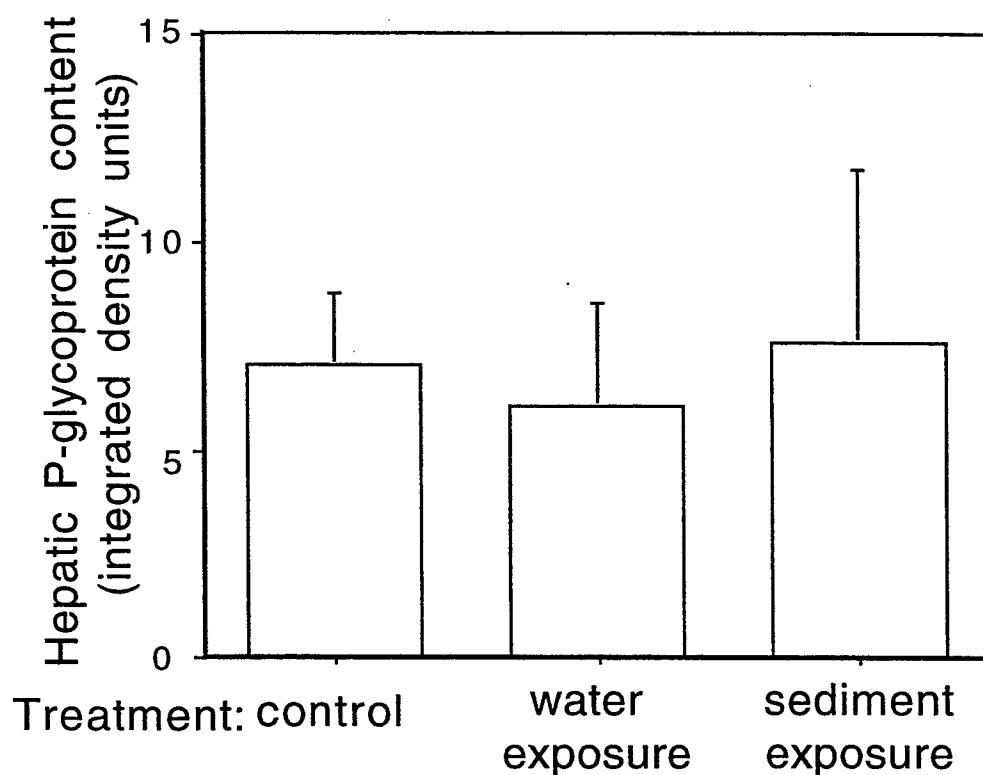Figure 2. Relative expression of P-glycoprotein in liver lysates of *F. heteroclitus* analyzed by Western blot using anti-P-glycoprotein mAb C219. Killifish were collected from either (A) the reference site at Scorton Creek or (B) the PCB contaminated site at New Bedford Harbor then maintained in the laboratory in clean water for up to 78 days. P-glycoprotein expression is reported as mean integrated density ± standard deviation as described in methods. (*) indicates significant difference from fish sampled on day 1 ($p < 0.05$). A value of 100 arbitrary units was assigned to the mean integrated density measured for a sample of 5 μg of a pooled liver lysate. Values in this experiment cannot be compared to arbitrary units used in the sediment exposure experiment.
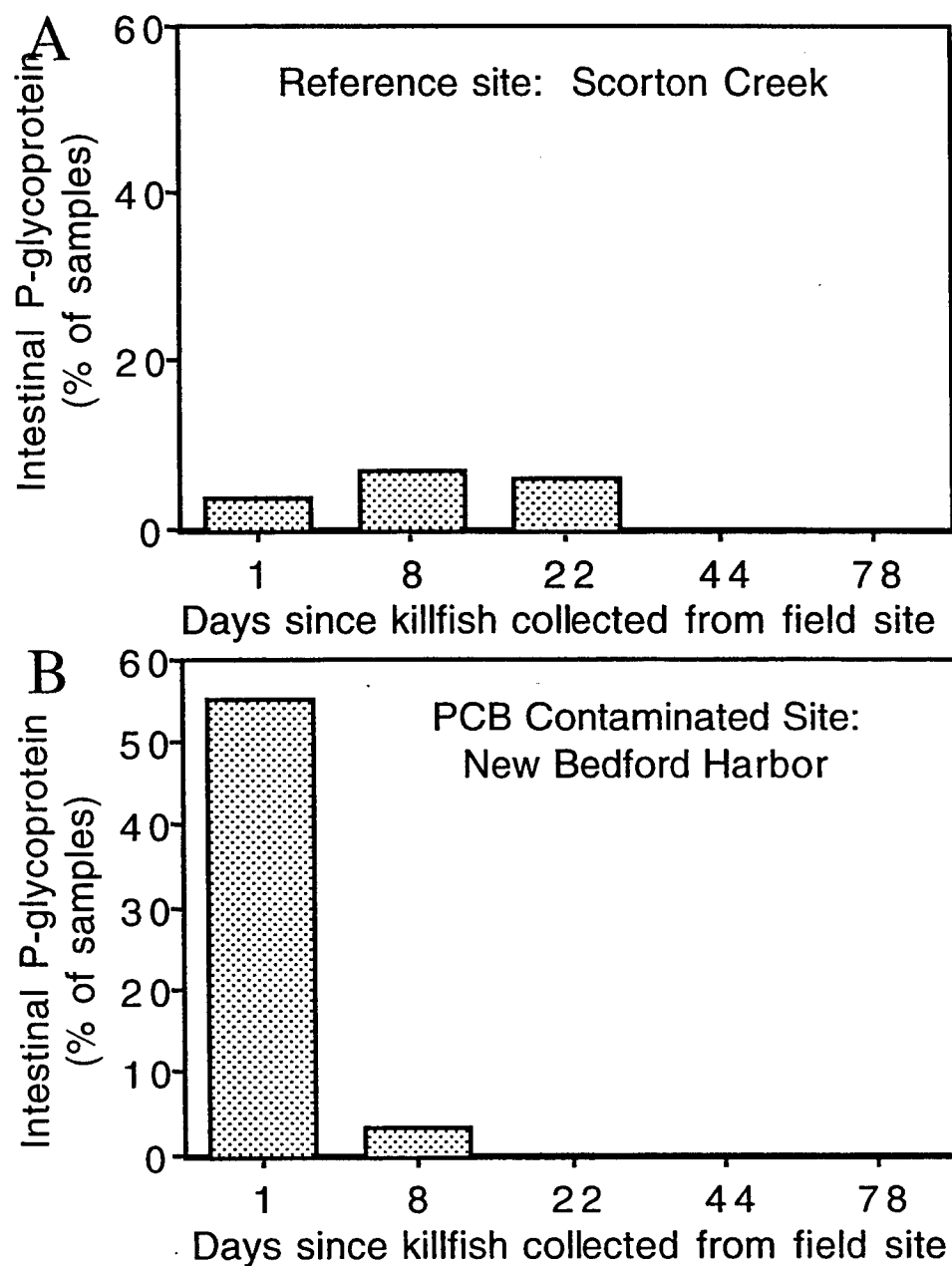
Figure 3. Percent of *F. heteroclitus* with P-glycoprotein detected in intestinal
epithelium by immunohistochemical analysis using anti-P-glycoprotein mAb
C219. Killifish were collected from either (A) the reference site at Scorton
Creek or (B) the PCB contaminated site at New Bedford Harbor then maintained
in the laboratory in clean water for up to 78 days.
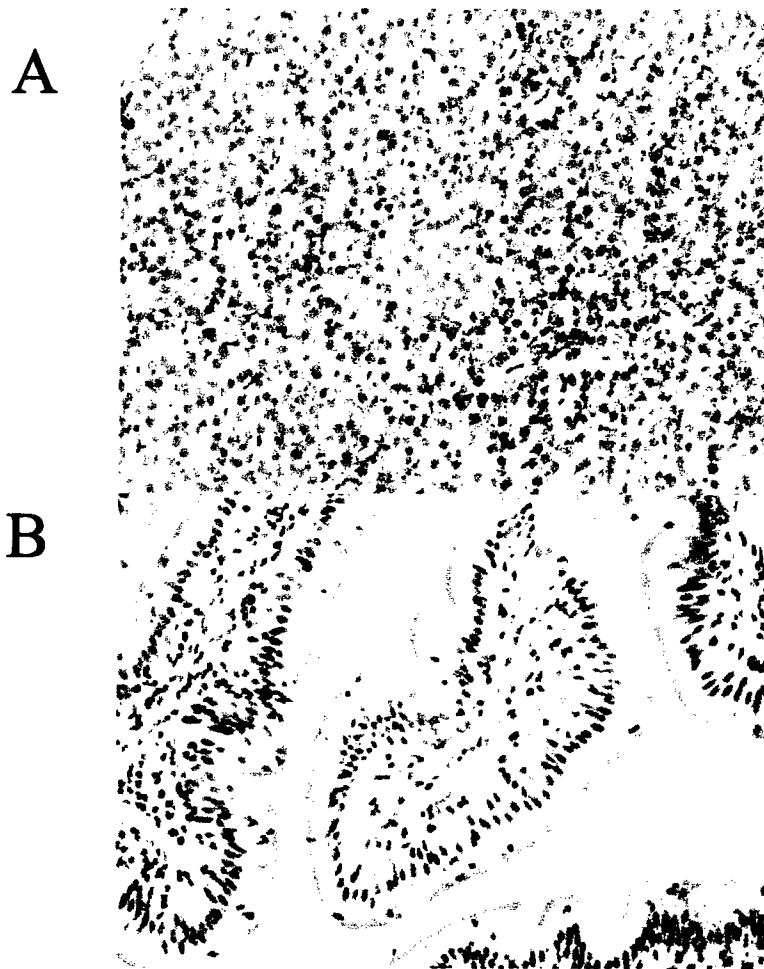
Figure 4. *F. heteroclitus* immunohistochemical results.
Specimens sampled one day after fish was collected
from the PCB contaminated site at New Bedford
Harbor. Red stain indicates immunoreaction with anti-
P-glycoprotein mAb C219. (A) In the liver, P-
glycoprotein is localized to the bile canaliculi. (B) In
the intestine, P-glycoprotein is localized to the
epithelium.

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Figure 5. Relative expression of P-glycoprotein in liver lysates of sediment exposed *F. heteroclitus* analyzed by Western blot using anti-P-glycoprotein mAb C219. Killifish were collected from a reference site at Scorton Creek and maintained in the laboratory in clean water for 6 months (i.e. depurated). Fish were then held in tanks with clean standing water alone or containing sediment from Scorton Creek for 7 days. Control fish were sampled from the depurated population at the start of the experiment. P-glycoprotein expression is reported as mean integrated density ± standard deviation as described in methods. A value of 10 arbitrary units was assigned to the mean integrated density measured for a sample of 10 µg of a pooled liver lysate. Values in this experiment cannot be compared to arbitrary units used in the depuration experiment.
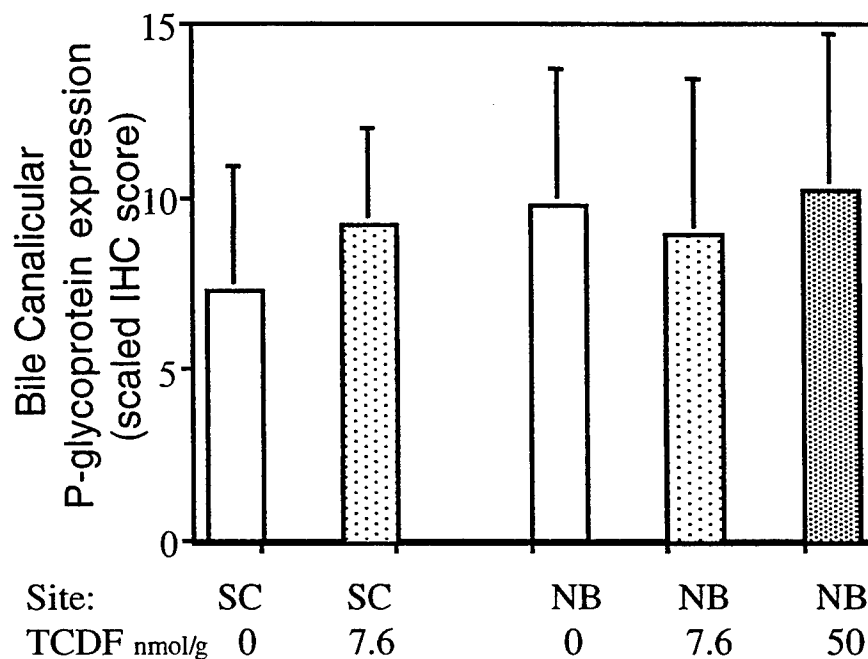
Figure 6. Relative expression of P-glycoprotein in bile canaliculi of 2,3,7,8-tetrachlorodibenzofuran [TCDF] exposed *F. heteroclitus*. Killifish were collected from a reference site (Scorton Creek [SC]) and PCB contaminated site (New Bedfore [NB], depurated in clean water in the lab for 17 months, then injected i.p.with at 7.6 or 50 nmol TCDF/g fish or vehicle (corn oil [0]). P-gp expression is reported as the mean scaled immunohistochemical (IHC) staining score ± standard deviation as described in methods.

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Chapter 6

Inhibition of Rhodamine B Transport in Liver, Brain, and Ovary by Cyclosporin A: Development of an *in vivo* Model for Analysis of P-glycoprotein Function.

Abstract

P-glycoproteins (P-gps) confer multidrug resistance in tumor cell lines; whether P-gps provide *in vivo* resistance against accumulation of xenobiotics in natural populations of vertebrates is the focus of this study. The purpose of this study was to characterize how P-gp affects the intracellular disposition and retention of a xenobiotic *in vivo*. We have developed a protocol for an *in vivo* assay, and applied this assay to evaluation of P-gp-mediated transport of a model substrate, rhodamine B (rhB), in multiple organs of killifish (*Fundulus heteroclitus*). *In vivo* inhibition of P-gps by the chemosensitizer cyclosporin A significantly decreased biliary efflux of rhB (84% lower than in rhB only fish), decreased accumulation in liver (29% lower) and gut (26% lower), and increased accumulation in blood (49% greater than in rhB only fish), brain (225% greater), and ovary (226% greater). No significant differences in rhB accumulation were observed in gill or kidney. We establish a non-mammalian vertebrate model for evaluating P-gp function *in vivo* at the organismal and population levels. The large inter-individual variability in P-gp activity that we observed may effect disposition and detoxification of xenobiotics and may be a determinant of individuals' differences in pharmacological response to drugs and toxicological effects of environmental contaminants that are P-gp substrates. Our results indicate that P-gps play a major role in transport of xenobiotics in fish, especially in liver, brain, and ovary.

Keywords: P-glycoprotein; multidrug resistance; multixenobiotic resistance; *in vivo* assay; fish; aquatic environment.

Introduction

Transmembrane P-glycoproteins function as energy dependent efflux flippases that prevent the cellular accumulation of a wide variety of moderately hydrophobic compounds. Known P-gp substrates include endogenous regulators such as glucocorticoids (Naito *et al.*, 1989; Ueda *et al.*, 1992), drugs and other natural products (Gottesman and Pastan, 1988), and environmental contaminants (Phang *et al.*, 1993; Cornwall *et al.*, 1995; Bain and LeBlanc, 1996). These transporters are located in tissues involved in secretion or absorption (e.g. liver, kidney, intestine, adrenal cortex), and in tissues that have tight junction blood barriers (e.g. blood-brain and blood-testes barriers) (Fojo *et al.*, 1987; Thiebaut *et al.*, 1987; Cordon-Cardo *et al.*, 1990; Sugawara, 1990). P-glycoproteins (P-gps) are responsible for certain multidrug resistance (MDR) phenotypes in tumor cell lines and in human cancer patients (Juliano and Ling, 1976; Gerlach *et al.*, 1986). Although P-gps have been intensively studied in relation to carcinogenesis using highly selected drug resistant cell lines and chemotherapeutic agents, less is known regarding multixenobiotic resistance in natural populations of organisms including humans. Studies conducted *in vitro* may not accurately reveal the role of P-gps *in vivo*. Characterization of P-gp function *in vivo* will allow a better understanding of how P-gp may influence the toxicological effects of its substrates on, for example, tissue disposition, interactions with other xenobiotics, and expression of xenobiotic inducible metabolizing enzymes. Understanding the effect of P-gp activity on xenobiotic disposition at the organismal level is important for evaluating, for example, the health risk of environmental contaminants to a human

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population. Model species must be selected for such studies when one is ethically prohibited from conducting this work in humans.

We developed an assay to detect P-gp-mediated transport of a potential xenobiotic substrate *in vivo* using a non-mammalian model, killifish (*Fundulus heteroclitus*), from a natural population. The purpose of this study was to use this assay to evaluate how P-gp affects the intracellular disposition and retention of a xenobiotic *in vivo*. Killifish were selected as the model study organism because P-gp-like genes (Cooper, 1996) and elevated expression of hepatic P-gps (Cooper *et al.*, 1999) have been described in this species, and populations of this fish have developed resistance to exposure to anthropogenic pollutants in their estuarine environment (Williams, 1994; Prince and Cooper, 1995a; Prince and Cooper, 1995b; Hahn, 1998; Nacci *et al.*, 1999; Elskus *et al.*, 1999). The toxin exposure history of humans may more closely resemble that of coastal fish, which are continually challenged by exposure to water-borne anthropogenic contaminants and natural product toxins, than inbred rodents maintained in laboratories and fed clean food. Both natural products and anthropogenic contaminants found in the aquatic environment appear to be substrates and/or inducers of P-gps in aquatic organisms (Cornwall *et al.*, 1995; Toomey *et al.*, 1996; Smital and Kurelec, 1998).

We investigated whether *in vivo* inhibition of P-gp by a chemosensitizer drug (cyclosporin A (CsA)) altered the accumulation of the fluorescent dye rhodamine B (rhB), a known P-gp substrate, in bile, liver, brain, ovary, gastrointestinal tract, blood, gill and kidney. Organs selected for examination are those in which P-gps may be involved in a barrier function or detoxification, and/or for which there is evidence of P-pg expression in fish by immunoblot, immunohistochemistry or *in vitro* transport assays (Hemmer *et al.*, 1995; Miller, 1995; Cooper *et al.*, 1996). The development of our *in vivo* assay in a small fish that is easy to maintain in large numbers (several 1000 individuals) in the laboratory permits analysis of larger sample sizes (>40 fish) for each experiment than are normally

economical for rodent studies. Large sample sizes are especially important because these fish have large individual variability in the magnitude of P-gp expression within a wild population (Bard *et al.*, 2000 in preparation) as have humans (Schuetz *et al.*, 1995a). Individual variability in P-gp activity may effect disposition and detoxification of xenobiotics and may be a determinant of individuals' differences in pharmacological response to drugs and toxicological effects of environmental contaminants which are P-gp substrates (Schuetz *et al.*, 1995b; Bain and LeBlanc, 1996). Such diversity in response cannot be examined in mdr knockout mouse models for obvious reasons.

Rhodamine dyes have been used extensively as probes to study MDR *in vitro* and *in vivo* (Neyfakh, 1988; Wang *et al.*, 1995; Kunihara *et al.*, 1998; Marques-Santos *et al.*, 1999). Cyclosporin A, one of the best studied MDR modulators, is known to be transported by P-gp and inhibit the efflux of other substrates both *in vitro* and *in vivo* (Slater *et al.*, 1986; Erlichman *et al.*, 1993). The *in vivo* inhibition of the secretion of rhodamine 123 (rh123), structurally similar to rhB, by CsA has been previously reported in rat liver and kidney (Kunihara *et al.*, 1998), rat brain (Wang *et al.*, 1995) and in mouse thymus and lymph nodes (Marques-Santos *et al.*, 1999). In mdr 1a and/or 1b knockout mice (Schinkel *et al.*, 1997) and in CF-1 mutant mice which are phenotypically similar to mdr1a knockouts (Lankas *et al.*, 1998) a lack of *in vivo* secretion of several P-gp substrates, including rhodamines, was observed in those organs which normally express P-gp in wildtype mice. Using an inexpensive and rapid assay, we evaluated the effect of P-gp-mediated transport on xenobiotic distribution *in vivo* in multiple organs and in large numbers of individuals from a natural population of vertebrates. Our results indicate that P-gps play a major role in transport of xenobiotics especially in liver, brain, and ovary.

Methods

Materials

Cyclosporin A and Rhodamine B (N-[9-(2-carboxyphenyl)-6-(diethylamino)-3H-xanthen-3-ylidene]-N-ethylethanaminium chloride) were purchased from Sigma (St. Louis, MO). Ringer's buffer consisted of 116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl2, 5 mM HEPES, pH 7.2.

Animal maintenance

Fundulus heteroclitus were collected in July, 1998 and July, 1999 from a putative clean field site (Scorton Creek, MA) 3-12 months prior to experiments. Previous chemical analysis has shown that polychlorinated biphenyl (PCB) body burden is low in Scorton Creek killifish (ΣPCB = 0.177 μg/g carcass dry weight) (Bello, 1999). Fish were maintained at Woods Hole Oceanographic Institution in flowing, filtered seawater at 15° C with aeration and thrice weekly were fed frozen brine shrimp and Tetramin flake fish food. Gravid females were selected for all experiments.

Dissection and sample preparation

Organs from 9 untreated fish were dissected and pooled in the following manner. The tail of the fish was severed by razor blade, fish were gently shaken to increase blood flow from the caudal vein, and heparinized glass capillary tubes were used to collect blood from the exposed caudal vein. Fish were sacrificed by cervical scission and the various organs removed and weighed: bile (the gall bladder was drained and discarded), liver, kidney, gastrointestinal tract (flushed with homogenizing buffer to remove gut contents), ovary, gill, brain, and blood. Each organ was homogenized in a buffer consisted of 50 mM Tris-HCl, 0.15 M KCl, pH 7.5. To provide sufficient sample volume for analysis,

the following ratios of organ weight to volume of homogenizing buffer were used: liver (1:5); kidney (1:19); gastrointestinal tract (1:5); ovary (1:3); gill (1:9); brain (1:10); blood (1:6); bile (1:8). Samples were stored in the dark at -40 C until used. Defrosted samples were processed in a glass homogenizer with a drive powered tissue grinder. Once homogenized, samples of organ homogenates were used immediately in the following experiments.

Characterization of rhodamine B fluorescence

For analysis of rhB fluorescence, 90 µl of each sample were loaded onto 96 well plates in triplicate. Ten µl of 5 µM rhB was added to each well for a final [rhB] = 0.5 µM/sample well, except for experiment (i) (refer to procedure below). A serial dilution of rhB in water or homogenizing buffer (0, 0.01, 0.05, 0.10, 0.50, 1.00 µM) was included on each plate as a standard. For experiment (i) and (ii), 100 µl of organ homogenates from untreated fish were loaded as "organ blanks" to account for native fluorescence of each organ. A cytofluor fluorescence plate reader was used to measure sample fluorescence within the range for rhB (ex: 530/25 nm; em: 590/35 nm). We conducted four experiments to characterize how rhB fluorescence is affected by the nature of the analyzed sample by varying the following qualities: (i) organ type, (ii) dilution of organ homogenate, (iii) density, (iv) pH.

(i) First, we examined if the type of organ homogenate influences rhB fluorescence (refer to In vitro protocol I). Six samples of each type of organ homogenate (i.e. liver, kidney, etc. prepared from untreated fish as described above) were loaded onto 96-well plates in triplicate. To each sample, 10 µl of serially diluted rhB was added to attain the following set of rhB concentrations for each organ homogenate: 0 µM, 0.01, 0.05, 0.10, 0.50, 1.0).

(ii) Second, we tested whether the dilution of organ homogenates affected rhB fluorescence (refer to In vitro protocol I). Organ homogenates were prepared from untreated fish as described above. These homogenates were serially diluted with additional homogenizing buffer in the following ratios of organ homogenate weight to volume of buffer (1:0, 1:1; 1:4, 1:9, 1:19) and were vortexed until the samples were well mixed.

(iii) Third, we examined the effect of sample density on rhB fluorescence (refer to In vitro protocol II). As lipid components can affect sample density, water soluble glycerol was selected as a test substance. Glycerol is a sugar alcohol that comprises the backbone of triglycerides which is the most abundant family of lipids and the major component of storage lipids in animal cells. Glycerol was serially diluted in homogenizing buffer: 1.25, 6.25, 12.5, 18.75, and 25% glycerol in buffer.

(iv) Finally, we tested the effect of pH on rhB fluorescence, aliquots of deionized water and the homogenizing solution (50 mM Tris-HCl, 0.15 M KCl) were prepared at a range of pH values (3.0, 5.0, 7.0, 8.0, 10.0 and 3.0, 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 10.0 respectively) (refer to In vitro protocol II).

In vivo **Assay: rhodamine B +/- cyclosporin A**

Fish were injected via the caudal vein, located in the tail along the backbone below the position of the caudal fin, with 0.3 µg rhB dissolved in Ringer's buffer per g fish with or without 10 µg/g CsA suspended in Ringer's buffer (refer to In vivo protocol). Fish were sacrificed 1 hour post-injection and for each fish its liver, kidney, gastrointestinal tract, ovary, gill, brain, blood, and bile were collected as described above. Organs and fluids were homogenized in the following ratios of organ weight to volume of homogenizing buffer: bile (1:12), liver (1:10), kidney (1:18), gastrointestinal tract (1:5), ovary (1:9), gill (1:16), brain (1:12), blood (1:9). Samples were homogenized as previously described. One hundred µl of each sample was loaded in triplicate onto a 96

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well plate and rhB fluorescence measured using a cytofluor fluorescence plate reader. Rhodamine B standards diluted in homogenizing buffer and organ blanks were included on each plate as described above.

To eliminate variability between scans with the plate reader, and to account for the quenching or augmentation of fluorescence due to the type of organ examined or its dilution, the following internal standardization procedure was developed. Loaded plates were scanned once, then 10 pmol of rhB was added to each sample well but not to standard curve or organ blanks, and the plate was scanned a second time. The fluorescence due to rhB in each sample is equal to the measured fluorescence of the organ homogenate minus the fluorescence of the appropriate organ blank. The equation of the standard curve (fluorescence versus concentration) was calculated and used to convert fluorescence in a sample to a *standardized value* (*) for both scan 1 and scan 2. The values obtained do not equate to rhB concentrations in organ homogenates because, as experiments with organ dilutions shown in this paper demonstrate, the standard curve of rhB in organs differs from that in homogenizing buffer. The difference in standardized values between scan 1 and scan 2 is due to the addition of the 10 pmol rhB. The incremental increase due to the addition of 10 pmol rhB in scan 2 is compared to the scan 1 value to calculate the true concentration of rhB in each sample homogenate. Using the dilution factor and organ mass, the concentration and total moles of rhB for all organs in each fish can be calculated as illustrated below. The data were analyzed in four ways as seen below: percent of total dose of rhB in each organ; concentration of rhB in each organ; percent change in average rhB concentration in each organ in CsA-treated fish compared to fish given rhB alone; and rhB concentration ratios of tissue-to-blood and bile-to-liver.

sample dilution factor = $\dfrac{\text{organ mass (g)}}{\text{organ mass (g) + added homogenizing buffer mass (g)}}$

[rhB] in organ (mol/g) = $\dfrac{(10^{-12}\text{ moles})}{(\text{scan 2}^* - \text{scan 1}^*)} \times \text{scan 1}^* \times \dfrac{1000\,\mu l}{100\,\mu l} \times \dfrac{1\,ml}{ml} \times \dfrac{1}{g} \times \dfrac{1}{\text{dilution factor}}$

moles rhB in organ = $[rhB]\,\dfrac{(\text{mol})}{g} \times \text{organ mass (g)}$

Percent of total dose in organ = $\dfrac{\text{moles rhB in organ}}{\text{moles rhB in injected dose}}$

Percent change in [rhB] = $\left\{ \dfrac{\text{(average [rhB] in organ of CsA-treated fish)}}{\text{(average [rhB] in organ of rhB only treated fish)}} \times 100\% \right\} - 100\%$

*indicates *standardized values* which were calculated as previously described

Statistical Analyses

Differences between rhB accumulation (concentration and percent of total dose) in each organ, and ratios of organ-to-blood and liver-to-bile from fish treated with or without CsA were statistically analyzed by one-way ANOVA using Fisher's protected LSD procedure using the SuperANOVA (Abacus Concepts) statistical program; $p \leq 0.05$ was accepted as significant. Using the same program, linear regression analysis was performed between rhB fluorescence and each of the following factors: organ homogenate dilution factor, rhB concentration in organ homogenates, glycerol content in buffer, and pH in buffer and water .

Results

Rhodamine B fluorescence in organ homogenates

The type of organ and the dilution of the organ homogenate both were found to affect the fluorescence measured in the preparations (Figure 1). The fluorescence due to 0.5 μM rhB in kidney and blood samples decreased greatly with increased organ homogenate concentration. Fluorescence in liver and bile homogenates decreased slightly, while fluorescence in gill and intestine increased slightly with increased proportion of organ in homogenate (Figure 1). In contrast, rhB fluorescence in ovary and brain homogenate increased greatly with increases in organ homogenate concentration. A 1% increase in the concentration of brain homogenates {(1 part brain)/(1 part brain + 99 parts buffer)}corresponded to an elevated rhB fluorescence of 743 relative units. A 1% increase in the concentration of ovary homogenates increased rhB fluorescence 1551 relative units.

When rhB was serially diluted in organ homogenates, the measured fluorescence differed from that of the same concentration of rhB in water, depending on organ type (Figure 2). The measured fluorescence for the rhB in ovary, brain, intestine, and gill was elevated compared to the standards in water. Curves for rhB fluorescence in bile and in liver and kidney homogenates were similar to that in water. The fluorescence of rhB in blood was depressed compared to rhB in water.

The unexpected result that rhB fluorescence was elevated in ovary and brain homogenates compared to its fluorescence in water prompted us to evaluate possible sources of this variation. We examined sample density and variable pH for effects on rhB fluorescence. As lipid components can affect sample density, glycerol was added to solutions of rhB in buffer. Increased percent of glycerol added to homogenizing buffer was associated with elevated rhB fluorescence (Figure 3). There is a linear relationship between rhB fluorescence and glycerol content with a slope of 98 relative rhB fluorescence units/percent glycerol. Increase in pH was associated with elevated fluorescence of rhB in

water (Figure 4). There is a linear relationship between rhB fluorescence and pH unit in water with a slope of 441 relative rhB fluorescence units/pH unit. However, no significant effect of pH on fluorescence was noted for rhB in homogenizing buffer. The level of increase in rhB fluorescence observed with elevated glycerol content and pH was not sufficiently large to account for the order of magnitude greater increase in fluorescence observed in the ovary and brain samples compared to rhB fluorescence in water. The presence of high levels of lipids or other components in brain and ovary samples might be responsible for the elevation in rhB fluorescence observed in homogenates of these organs.

In vivo Assay: rhodamine B +/- cyclosporin A

To overcome the problem of organ-specific effects on rhB fluorescence, a two-scan method was developed in which an internal standard of 10 pmol rhB was added to each sample after scan 1, and the incremental increase in fluorescence in scan 2 was used to calculate the original rhB concentration of samples. In experiment 1, fish were injected intravenously with rhB *in vivo* and 1 hour later the organ distribution of rhB was analyzed as a percent of total dose of rhB detected in each organ (Figure 5). In fish treated only with rhB, ~48% of the rhB injected was detected in the sampled organs and bile. Presumably, the remaining ~52% of rhB dose was distributed in the blood and throughout the carcass of the animal, while a portion may have been excreted, for example, across the gills or into the lumen of the gut. The highest proportion of injected rhB was detected in liver (30% of total dose), followed by gut (9.7%), bile (4.5%), ovary (1.4%), kidney (1.3%), gill (1.1%) and brain (0.09%). The concentration (nmol/g or μmol/L) of rhB in organs of these fish in decreasing order ranked liver > bile > kidney > gut > gill > blood > ovary > brain (Figure 6).

The co-administration of CsA and rhB in experiment 1 resulted in only 37% of the total dose of rhB being recovered in examined organs and bile. The most dramatic changes

in the distribution of the rhB when combined with CsA treatment compared to that in fish given rhB alone were decreases in liver (which contained 21% of total dose) and bile (0.7%), and increases in ovary (5%) and brain (0.3%) (Figure 5). In the rhB plus CsA-treated fish, the order of rhB concentration in organs also was altered, with rhB concentrations in liver > kidney > ovary > blood > gut > gill > bile > brain (Figure 6).

Comparing, the percent change in rhB concentration in organs of fish receiving rhB and fish treated also with CsA (Figure 7) showed that CsA-treated fish had significantly ($p<0.05$) lower concentrations of rhB in bile (83% less than in bile of rhB only fish), liver (29% less) and gut (26% less). In contrast, co-administration of CsA increased the rhB concentrations in blood (49% greater than in rhB only fish), brain (225% greater) and ovary (226% greater). No significant changes in rhB levels were detected in gill and kidney when CsA was administered together with rhB.

We examined rhB concentrations in whole blood rather than plasma in our experiment, as others have demonstrated that in rodents rhB concentration in whole blood is similar to that in plasma (de Lange *et al.*, 1998). The ratios of rhB concentration in brain or ovary to those in blood were significantly elevated in CsA-treated fish compared to the ratios in fish only given rhB (Table 1). The rhB concentration ratio of gut/blood declined slightly (40%) but significantly in CsA-treated fish. No differences with CsA treatment were observed in ratios of rhB concentrations in kidney/blood or gill/blood. The liver/blood ratio was slightly lower but not statistically significantly different ($p<0.06$) while the bile/liver ratio was significantly decreased in CsA-treated fish.

Experiment 1 included 19 fish per treatment. High variability in rhB concentration was observed in every organ, bile, and blood between individuals within each treatment groups (Figure 5 and 6). To test the reproducibility of the results of experiment 1, an additional two experiments were conducted, but using smaller numbers of fish (N=6 per treatment) and sampling only bile and brain. Values for rhB concentration and percent of

total dose are reported in Table 2. Experiments 2 and 3 yielded results similar to experiment 1, with the rhB concentration in bile of fish given rhB plus CsA being 86% and 94% lower than in fish receiving only rhB, and concentrations in brain being 158% and 263% greater than in brains of fish receiving only rhB (Figure 8).

Discussion

We have developed a protocol for an *in vivo* assay for P-gp function, and applied this assay to a non-mammalian model, the killifish *F. heteroclitus*. Previous studies of P-gp function *in vivo* have been carried out in mammals (Wang *et al.*, 1995; Kunihara *et al.*, 1998; de Lange *et al.*, 1998; Marques-Santos *et al.*, 1999). A decrease in secretion of rh123 associated with a lack of P-gp function was assessed in one or two organs in each study: brain (Wang *et al.*, 1995; de Lange *et al.*, 1998), kidney an d liver (Kunihara *et al.*, 1998), and thymus and lymph nodes (Marques-Santos *et al.*, 1999). Using the assay described here, we assessed P-gp function in multiple organs (liver, gut, gill, kidney, brain, and ovary) and evaluated P-gps role in excretion of substrates into bile.

During development of this assay, we observed that rhB fluorescence was strongly affected by homogenates of some organs. Although rhB fluorescence in bile and homogenates of kidney and liver were similar to fluorescence in water, the fluorescence was quenched in blood. In contrast, rhB fluorescence was moderately elevated in homogenates of intestine and gill and greatly elevated in homogenates of ovary and brain, compared to rhB fluorescence in water. The physicochemical conditions which account for quenching or augmentation of rhB fluorescence in these organs are unknown. The sample density (using glycerol content as a model substance) and pH were investigated as two possible variables affecting rhB fluorescence. Elevated glycerol content and increased pH in water were associated with elevated rhB fluorescence. However, there was not a significant relationship between rhodamine fluorescence and pH in the buffer used to

homogenize organs. The results suggest that the presence of high levels of triglycerides or other components in brain and ovary may be responsible for the augmentation of rhB fluorescence compared to the fluorescence in water. Our goal was to develop a method to relate rhB fluorescence to rhB concentration in multiple organs. As described in the Results, use of an internal standard overcame the problem of organ specific effect on rhB fluorescence.

In the experiments employing a known P-gp inhibitor, CsA, we examined organs that have previously been shown to express P-gps by immunohistochemical or *in vitro* functional data in fish (liver, intestine, kidney) (Hemmer *et al.*, 1995; Miller, 1995; Cooper *et al.*, 1996), or that might be expected to have an absorptive, blood-organ, or other barrier function (gill, brain), as well as the ovary. Rhodamine B and CsA are thought to be noncompetitive substrates which each interact separately with one of the three different P-gp binding sites identified to date (Shapiro and Ling, 1997; Shapiro and Ling, 1998; Shapiro *et al.*, 1999). The exact mechanism by which CsA binds to and preferentially inhibits P-gp over other xenobiotic transporters is unknown but evidence suggests that CsA disrupts P-gp function by modifying cell membrane fluidity (Fricker, 2000 personal communication). Nevertheless, CsA is thought to specifically interfere with P-gps. The effects of CsA on rhB disposition were most evident in liver, brain, and ovary. These are discussed below along with the potential involvement of P-gp in the kidney.

Liver and bile

In all three *in vivo* experiments, CsA treatment was associated with a significant decrease (84% to 94% lower than in rhB only fish) in rhB concentration in bile (Figure 8). Depression of rhB levels in bile by CsA treatment is consistent with the hypothesis that transport of rhB from liver to gall bladder is inhibited. Cyclosporin A has been shown to decrease the *in vivo* biliary excretion of rh123 in rats (Kunihara *et al.*, 1998), and non-

competitively inhibit the biliary secretion of rh123 in isolated perfused livers of a mutant rat strain (TR-) that is deficient in the canalicular multispecific organ anion transport system (cMOAT/MRP2) (Stapf *et al.*, 1994). Rhodamine B is mainly metabolized by glucoronidation and both rhB and its glucuronide metabolite are found in bile. The major transport mechanism of unconjugated rhB in liver is P-gp-mediated while the glucuronide conjugate is transported by cMOAT/MRP2 (Stapf *et al.*, 1994). Both in mammals (Stapf *et al.*, 1994) and in killifish isolated renal proximal tubules (Masereeuw *et al.*, 1996) CsA has been shown to inhibit P-gp-mediated transport preferentially over other transport mechanisms including cMOAT/MRP2. Thus, the effect of CsA in the fish here suggests the involvement of P-gps.

Inhibition of excretion of rhB into bile could lead one to expect an increase in rhB content in liver. In contrast to that expectation, a slight decrease in rhB accumulation was observed in the liver upon CsA treatment. Although this decrease is statistically significant, there is no significant difference between rhB concentration ratios of liver/blood in fish treated with rhB only compared to fish treated with rhB and CsA. Thus there is no strong e vidence for the presence of a CsA-sensitive rhB uptake mechanism in the liver. Furthermore, the large interindividual variability observed in this experiment leads us to have less confidence in small changes in concentration than in the several fold differences seen in other organs. Similar results for the liver were previously observed during an *in vivo* study of mice exposed to the P-gp substrate ivermectin and CsA (Kwei *et al.*, 1999). The concentration of ivermectin was slightly, though not significantly, lower in liver of CF-1 mice (a stock naturally deficient in *mdr1a*) compared to wildtype mice (Kwei *et al.*, 1999). In our study, the significant decrease in rhB concentration ratios of bile/liver in CsA plus rhB treated fish compared to rhB only treated fish, and the similar liver/blood ratios for both treatment groups, suggests that CsA decreases biliary secretion of rhB without affecting rhB uptake into the liver. In our study, the similar rhB concentration

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ratios of liver/blood in both fish given only rhB and those given rhB and CsA, and the significant decrease in bile/liver ratio in treated fish suggests that CsA decreases biliary secretion of rhB without affecting rhB uptake into the liver. These results are consistent with both *in vitro* and *in vivo* studies in rodents which showed no change in rh123 accumulation in the liver upon P-gp inhibition (Stapf *et al.*, 1994; Kunihara *et al.*, 1998).

Kidney

CsA did not significantly alter accumulation of rhB in kidney in our study. Our results may be explained by *in vitro* studies of *Fundulus* renal proximal tubules that show that although P-gp is involved in efflux of substrates into the lumen of the tubule, no change is observed in the cellular accumulation of P-gp substrates upon inhibition with CsA (Miller, 1995; Schramm *et al.*, 1995). These results are consistent with *in vitro* studies showing that rh123 secretion in the isolated perfused rat kidney is not inhibited by CsA (Masereeuw *et al.*, 1997). The majority of renal rh123 efflux appears to be mediated by a renal organic cation carrier with P-gp playing a minor role (Masereeuw *et al.*, 1997). Thus, cellular uptake of P-gp substrates in the kidney is by simple diffusion and the steady-state cellular accumulation is affected little by substrate efflux into the lumen (Miller *et al.*, 1998). However, Kunihara found that *in vivo* renal secretory clearance of rh123 was reduced 37% in CsA treated rats, indicating that rh123 is in part secreted via P-gp in renal proximal tubular cells (1998). The results from Masereeuw, Miller, and Kunihara suggest that in the kidney, rhB is secreted by P-gp and other transport systems and although kidney cellular fluorescence is unaffected by CsA as in our study, P-gp-mediated renal secretion is moderately inhibited.

Brain

In our *in vivo* studies, the concentrations of rhB detected in brain of fish given rhB but not CsA was very small (ranging from 0.09 to 0.19 nmol/g). Cyclosporin A treatment stimulated small absolute changes in rhB accumulation in brain (0.28 to 0.69 nmol/g), however the relative changes were large, with 158% to 263% greater rhB concentrations in fish given rhB and CsA than in those given rhB alone. Furthermore, rhB concentration ratios of brain/blood increased in CsA-treated fish suggesting that elevated rhB in brain is due to a partial disruption of the blood-brain barrier by CsA rather than a change in blood supply of rhB to the brain. Although P-gp has not yet been identified by immunohistochemistry in fish brain, in mammals P-gp is known to be localized to the luminal side of the capillary endothelial cells that comprise the blood-brain barrier (Cordon-Cardo *et al.*, 1989). We suggest that the CsA-sensitive transport activity observed in the brain is due to P-gps involvement in the blood-brain barrier in fish.

A study of freely moving rats using intracerebral microdialysis coupled with on-line HPLC found that accumulation of rh123 also increased in brain upon CsA treatment (Wang *et al.*, 1995). As in our study, the rats given rh123 and CsA had small absolute increases in brain rh123 levels (0.59% brain/plasma distribution coefficient in rats given only rh123 versus 2.17% in CsA-treated rats) which represented large relative changes in brain exposure (3 to 4 fold increase). Similarly, a four-fold increase in rh123 accumulation was observed in whole brain homogenates of mdr1a (-/-) mice compared to wildtype (de Lange *et al.*, 1998). *In vivo* microdialysis did not reveal any differences in rh123 accumulation in different parts of the brain (de Lange *et al.*, 1998).

P-glycoprotein inhibitors such as CsA are known to elevate drug plasma levels (Stiff *et al.*, 1995; Toffoli *et al.*, 1997). The rat microdialysis technique employed by Wang *et al.* (1995) permitted the separation of rh123 levels in residual tissue blood from those in the brain tissue. Such separation was not possible with our assay of whole brain

homogenates. However, the fact that in fish given rhB and CsA, rhB concentration increased more in brain (225% greater than in fish given only rhB) than in blood (49% greater than in fish given only rhB), suggests that elevated rhB in brain was not simply due to increased rhB levels in interstitial blood.

The blood-brain barrier of fish is generally considered less efficient than that of mammals, and fish have been shown to accumulate higher levels of polychlorinated biphenyls (PCBs) in the brain compared to other species (Bachour *et al.*, 1998). However, highly lipophilic PCBs are probably not P-gp substrates. Our study is the first report of P-gp involvement in the teleost blood-brain barrier. That rhB concentration in brain was several fold less than that in blood either with or without presumed inhibition by CsA suggests that the blood-brain barrier in fish may involve transport systems in addition to P-gp.

Ovary

In gravid female fish, the addition of CsA significantly elevated rhB accumulation in ovary (226% greater rhB concentration in CsA treated fish compared to fish given only rhB). That the of rhB concentration ratio of ovary/blood was significantly increased in CsA-treated fish suggests that the elevated rhB concentration in ovary is not due simply to increased rhB levels in blood passing through the ovary. Although P-gps have not been identified by immunohistochemistry in fish gonadal tissue, we suggest that the CsA-sensitive transport activity observed in the ovary is a measure of P-gp activity. In mammals, P-gp expression has been localized to both the luminal and abluminal membranes of the ovarian endothelium (Stewart *et al.*, 1996.) Although a blood-testis barrier is found in the seminiferous tubules formed by tight junctions between Sertoli cells (Fawcett *et al.*, 1970), neither testicular nor ovarian capillaries form a diffusion barrier against serum proteins (Gerdes *et al.*, 1992; Everett and Simmons, 1958; Kormano, 1969;

Mancini *et al.*, 1965). Thus, Stewart (1996) suggests that by definition a blood-ovary barrier does not exist. However the presence of ovarian P-gp on both luminal and abluminal faces of the endothelium suggests that P-gp may prevent endothelial cells themselves from accumulating moderately hydrophobic compounds. P-glycoproteins expressed in the reproductive system of mammals (testes, ovary, pregnant uterus, and placenta) are thought to transport steroid hormones, as well as excrete xenobiotics presumably to protect maturing sperm, eggs, and fetus from toxic exposure (Baas and Borst, 1988; Arceci *et al.*, 1990; MacFarland *et al.*, 1994; Nakamura *et al.*, 1997; Lankas *et al.*, 1998). Our results provide the first in vivo evidence for Pgp function in ovary, and suggest that in fish, P-gp may serve a similar protective role in the ovary.

Conclusion

In conclusion, we developed an inexpensive, simple, fast *in vivo* assay to measure P-gp activity in multiple organs in a vertebrate model species. In our experiments, CsA dramatically decreased the biliary efflux of rhB and strikingly increased accumulation in brain and ovary. These results are consistent with an expected inhibition of P-gp. These data suggest that in addition to hepatic transport, P-gp is an important mechanism of xenobiotics resistance in tissues such as the brain, ovary, and possibly developing oocytes that may be particularly sensitive to the accumulation of toxin compounds.

The high individual variability of P-gp expression seen in natural populations of fish is more similar to the distribution of P-gp expression that one might expect in a heterogeneous human population than in inbred rodent stocks. In the environment, fish that are continually challenged by exposure to water-borne anthropogenic contaminants and natural product toxins may be a good vertebrate model system to study the *in vivo* function of P-gp as a multixenobiotic resistance mechanism at the organismal and population levels.

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In vitro protocol I

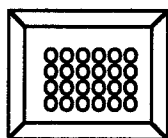Experiment 1 : Effect of organ type on fluorescence
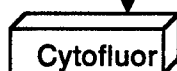
Untreated fish

Dissect organs/fluids

Dilute with buffer (organ weight:buffer volume) & homogenize

Liver	bile	kidney	gut	ovary	gill	brain	blood
1:5	1:8	1:19	1:5	1:3	1:9	1:10	1:6

Load 6 samples of 90 µl of each organ homogenate on a multiwell plate

 Add a serial dilution of rhB to each set of six samples
10 ul of rhB at 0 µM, 0.01, 0.05, 0.10, 0.50, 1.0

 Measure fluorescence

Experiment 2: Effect of dilution of organ homogenate on fluorescence

Untreated fish

Dissect organs/fluids

Dilute with buffer (organ weight:buffer volume) in the following ratios
of organ weight to volume buffer (1:0, 1:1, 1:4, 1:9, 1:19)Homogenize
and load 90 µl of each organ samples set on multiwell plate
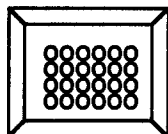
 Add 10 ul of 5 µM rhB to each sample well

 Measure fluorescence

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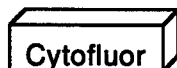
In vitro protocol II

Experiment 3: Effect of sample density on fluorescence

Dilute glycerol in buffer (1.25% glycerol, 6.25, 12.5 18.75, 25)

Load 90 µl of each set of glycerol-buffer solution
in triplicate to multiwell plate

Add 10 ul of 5 µM rhB to each sample well
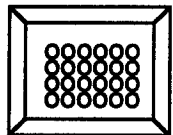
Cytofluor   Measure fluorescence
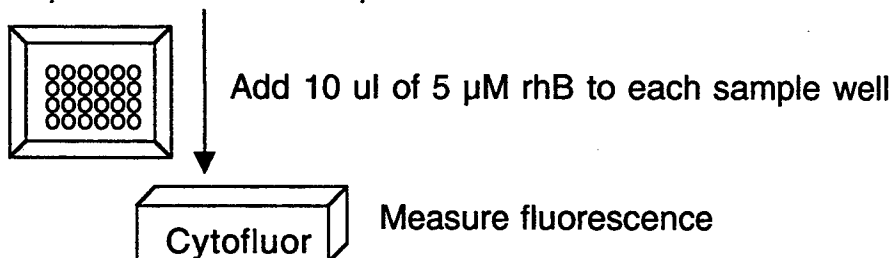
Experiment 4: Effect of pH on fluorescence

Prepare a set of aliquots of deionized water at a range of pH (3.0,
5.0, 7.0, 8.0, 10.0)
Prepare a set of aliquots of homogenizing solution (50 mM Tris-
HCl, 0.15 M KC) at a range of pH (3.0, 4.0, 5.0, 6.0, 6.5, 7.0,
7.5. 8.0, 8.5, 9.0, 10.0)

Load 90 µl of each set of aliquots in triplicate on a multiwell plate

Add 10 ul of 5 µM rhB to each sample well

Cytofluor   Measure fluorescence
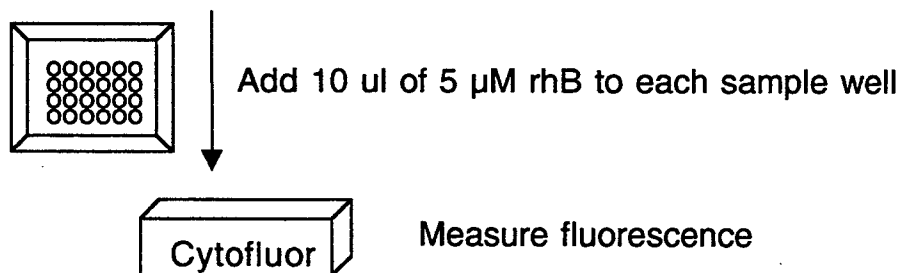
In vivo protocol

i.v. injection to caudal vein
control: rhB (0.3 µg/g fish)
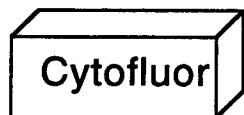+CsA: rhB (0.3 µg/g fish) + CsA (10 µg/g fish)

↓ 1 hour

Dissect organs/fluids
Dilute with buffer (organ weight:buffer volume) & homogenize

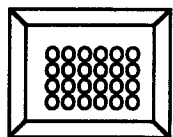| Liver	bile	kidney	gut	ovary	gill	brain	blood
1:10	1:12	1:18	1:5	1:9	1:16	1:12	1:9

Load 100 µl of each organ homogenate in triplicate on multiwell plate. Include rhB standards and organ homogenates from untreated fish for sample blanks

Cytofluor Measure fluorescence (Scan 1)

Add 10 pmol rhB to each sample
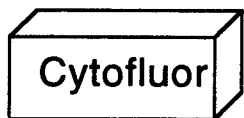
Cytofluor Measure fluorescence (Scan 2)

scan 2- scan 1=Δ fluorescence = 10 pmol rhB
Calculate: [rhB]
        % total dose rhB
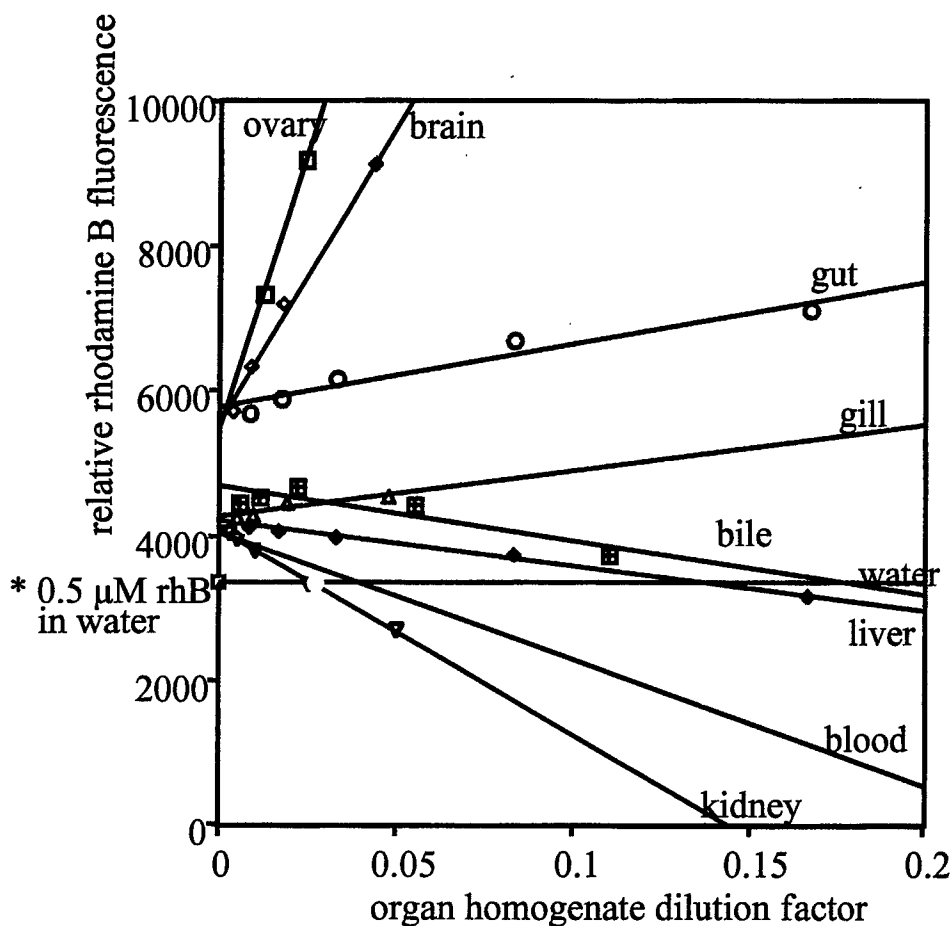   % Δ[rhB] between treatments

Figure 1. The effect of the dilution of organ homogenate on rhodamine B fluorescence. Rhodamine B fluorescence was measured in samples of various organ homogenates serially diluted with buffer and had 0.5 μM rhodamine B added to each sample. The organ homogenate dilution factor=(organ mass)/(organ mass + total buffer mass).

□ ovary r^2 = 1.000; ◇ brain r^2 = 0.992; ○ gut r^2 = 0.934;
▲ gill r^2 = 0.851   ⊞ bile r^2 = 0.808; ◆ liver r^2 = 0.994;
blood r^2 = 0.994; ▽ kidney r^2 = 0.996
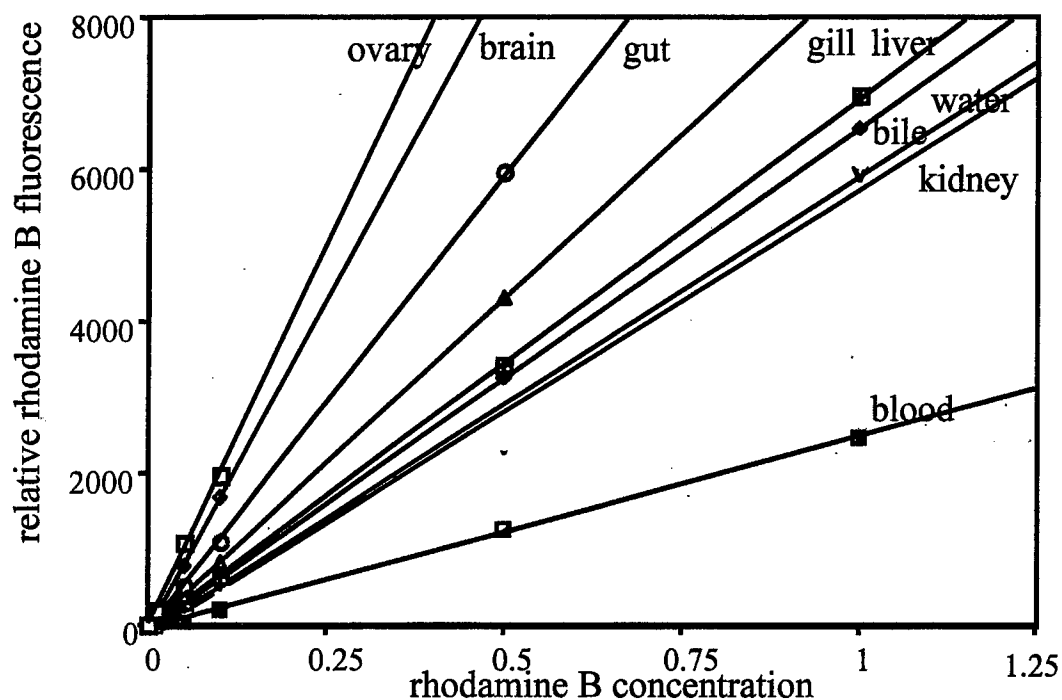*□ fluorescence of 0.5 uM rhodamine B in water

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Figure 2. The effect of organ type on rhodamine B
fluorescence. Rhodamine B fluorescence was measured in
samples of various organ homogenates to which was added
different concentrations of rhodamine B.

□ovary r^2 = 0.997; ◇brain r^2 = 0.999; ○gut r^2 = 1.000;
▲gill r^2 = 1.000; ⊞liver r^2 = 1.000; ◆bile r^2 = 1.000;
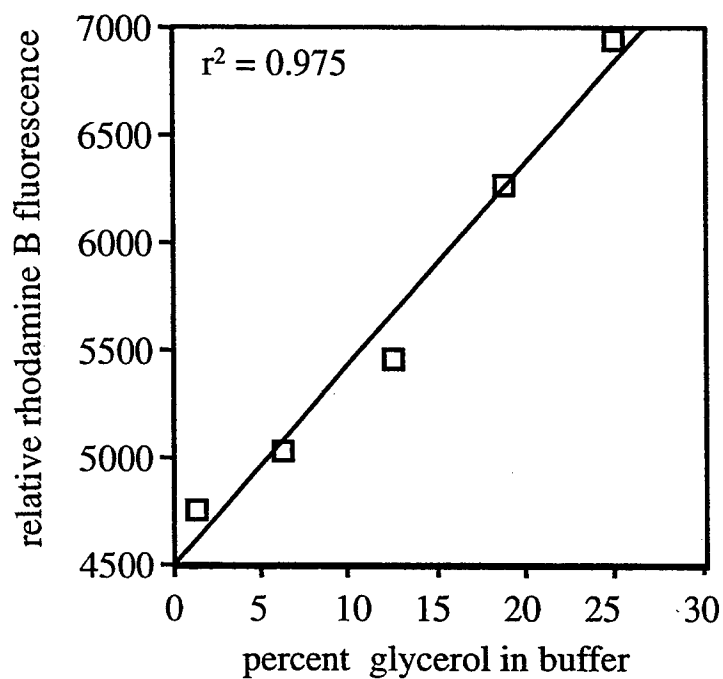water r^2 = 0.990; ▽kidney r^2 = 0.990; ▣blood r^2 = 0.999

Figure 3. The effect of lipid constituent (glycerol) content on rhodamine B fluorescence. Fluorescence was measured in aliquotes of homogenizing buffer with rhodamine B concentration of 0.5 µM and to which were added different proportion of glycerol.
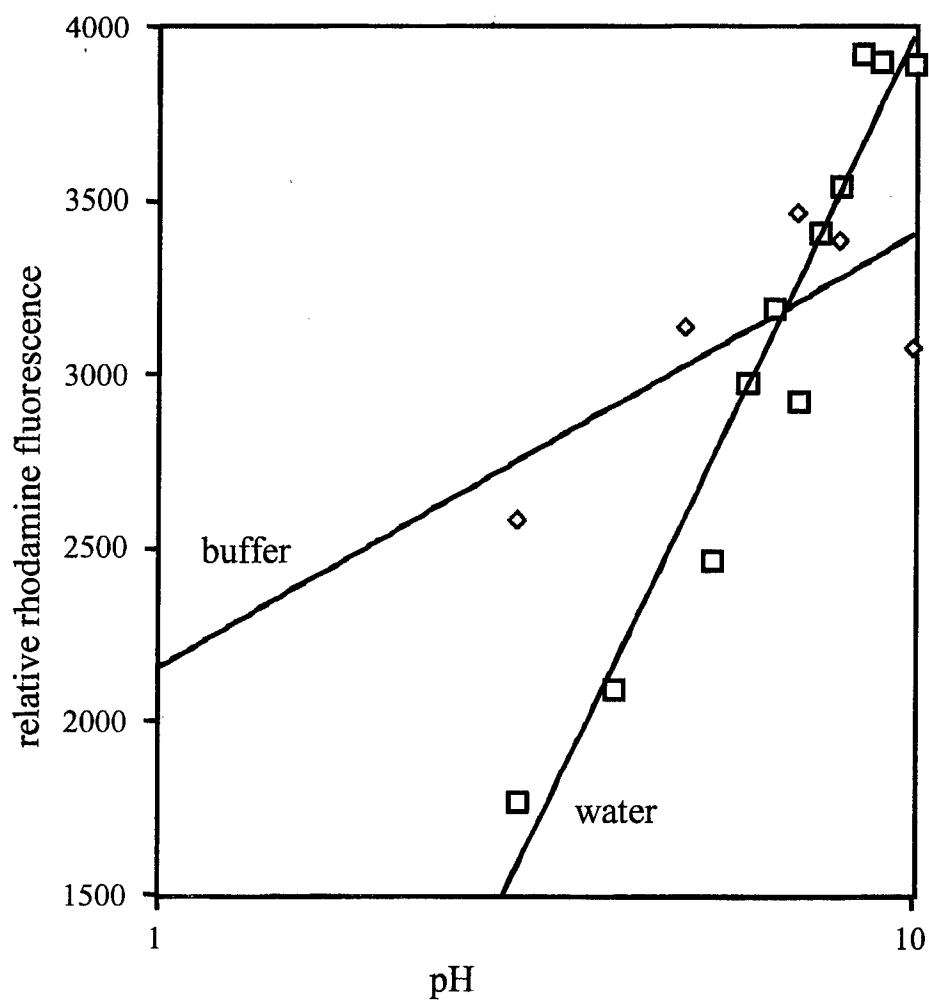
Figure 4. The effect of pH on rhodamine B fluorescence. Aliquots of deionized water and homogenizing buffer were prepared at a range of pH values. Each aliquot had a rhodamine B concentration of 0.5 μM.

□   0.5 μM rhB in water $r^2 = 0.938$
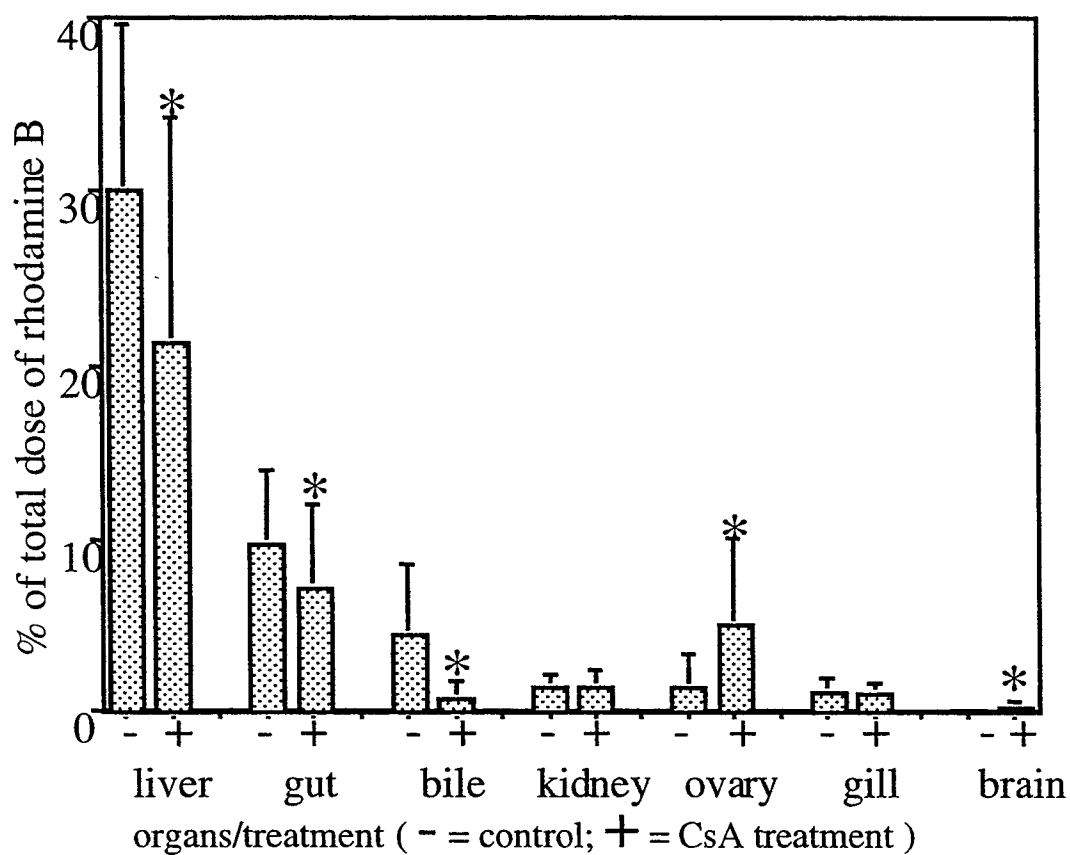
◇   0.5 μM rhB in homogenizing buffer $r^2 = 0.540$

Figure 5. Percent of total dose of rhodamine B detected in organs of treated fish (0.3 µg/g rhB + 10 µg/g cyclospor in A) compared with control fish (0.3 µg/g rhB) in experiment 1 (N=19 fish/treatment). Values are reported as the percent of the original total dose of rhB ± standard deviation. * indicates significant difference from control (p≤0.05).
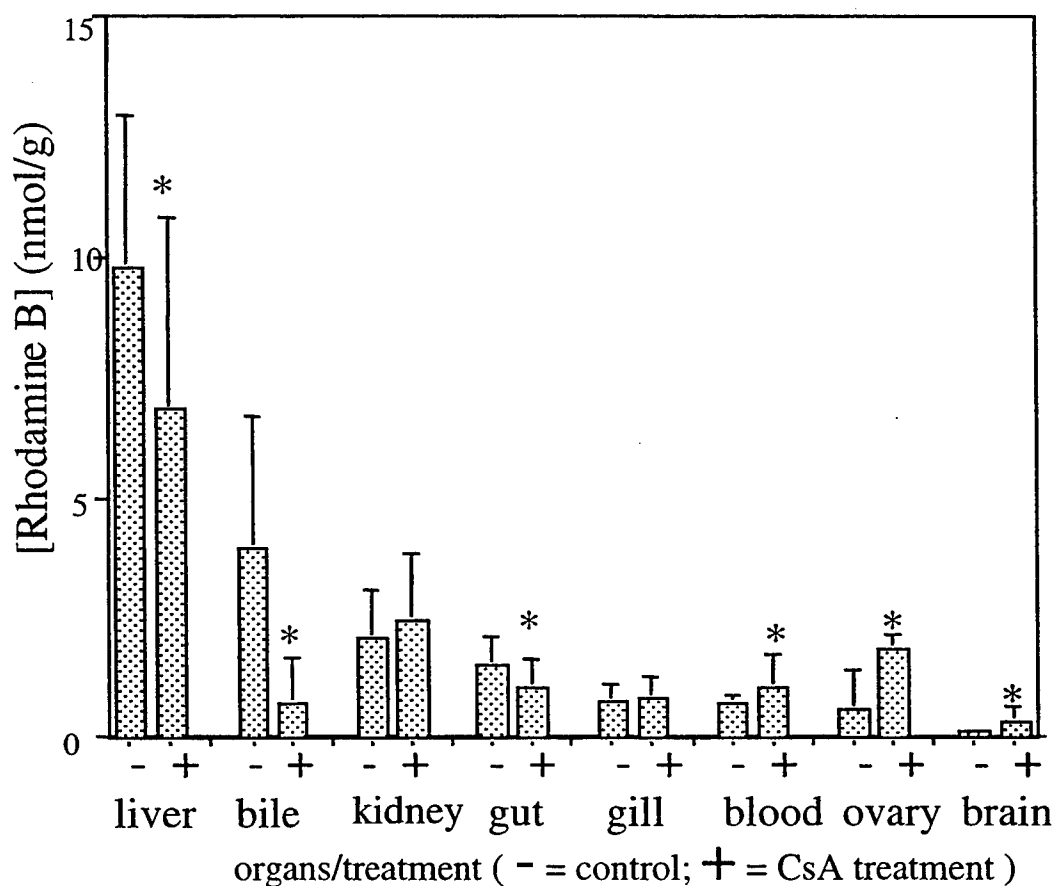
Figure 6. Concentration of rhodamine B detected in organs of treated fish (0.3 µg/g rhB + 10 µg/g cyclosporin A) compared with control fish (0.3 µg/g rhB) in experiment 1 (N=19 fish/treatment). Values are reported as µM rhB ± standard deviation. * indicates significant difference from control (p≤0.05).

Figure 7. Percent change in average rhodamine B concentration in organs of treated fish (0.3 μg/g rhB + 10 μg/g cyclosporin A) compared with control fish (0.3 μg/g rhB) in experiment 1 (N=19 fish/treatment). No change in average [rhB] in CsA-treated fish compared to control fish is indicated by a value of 0% while an increase is > 0% and a decrease < 0%. * indicates that average [rhB] in organs of rhB + CsA-treated fish is significantly different from that of control rhB only fish (p≤0.05).
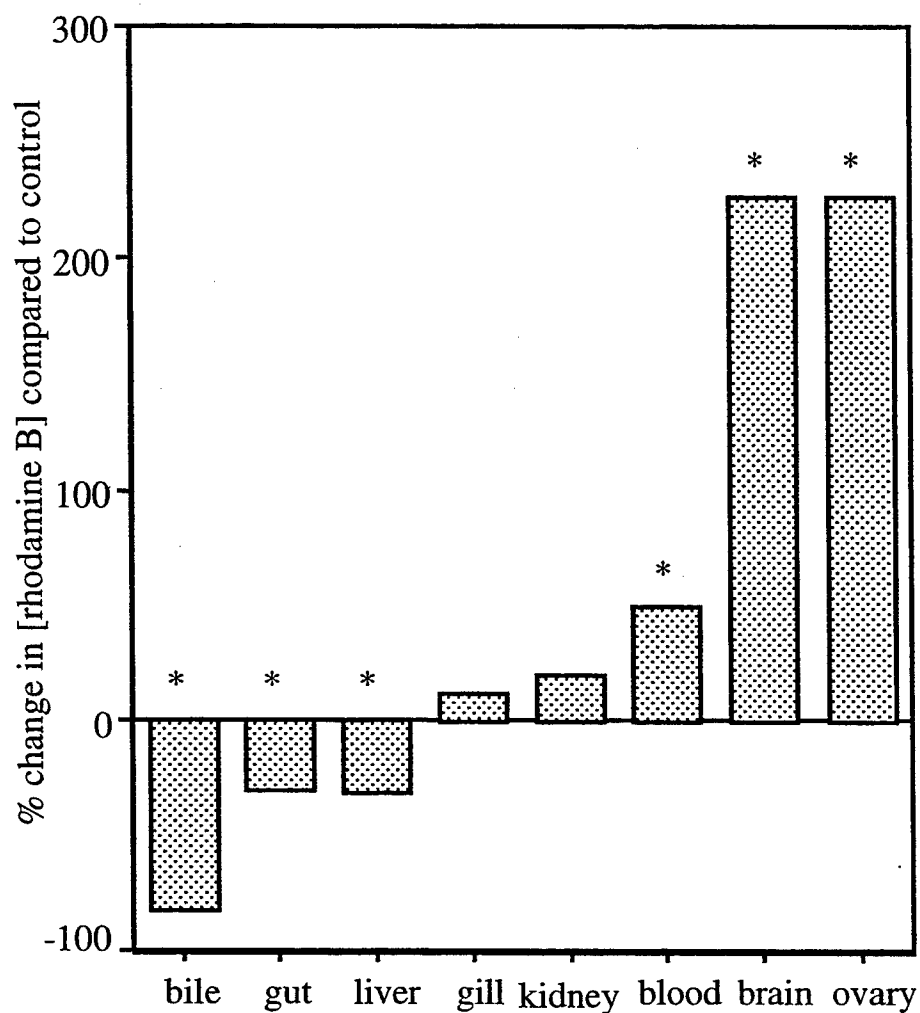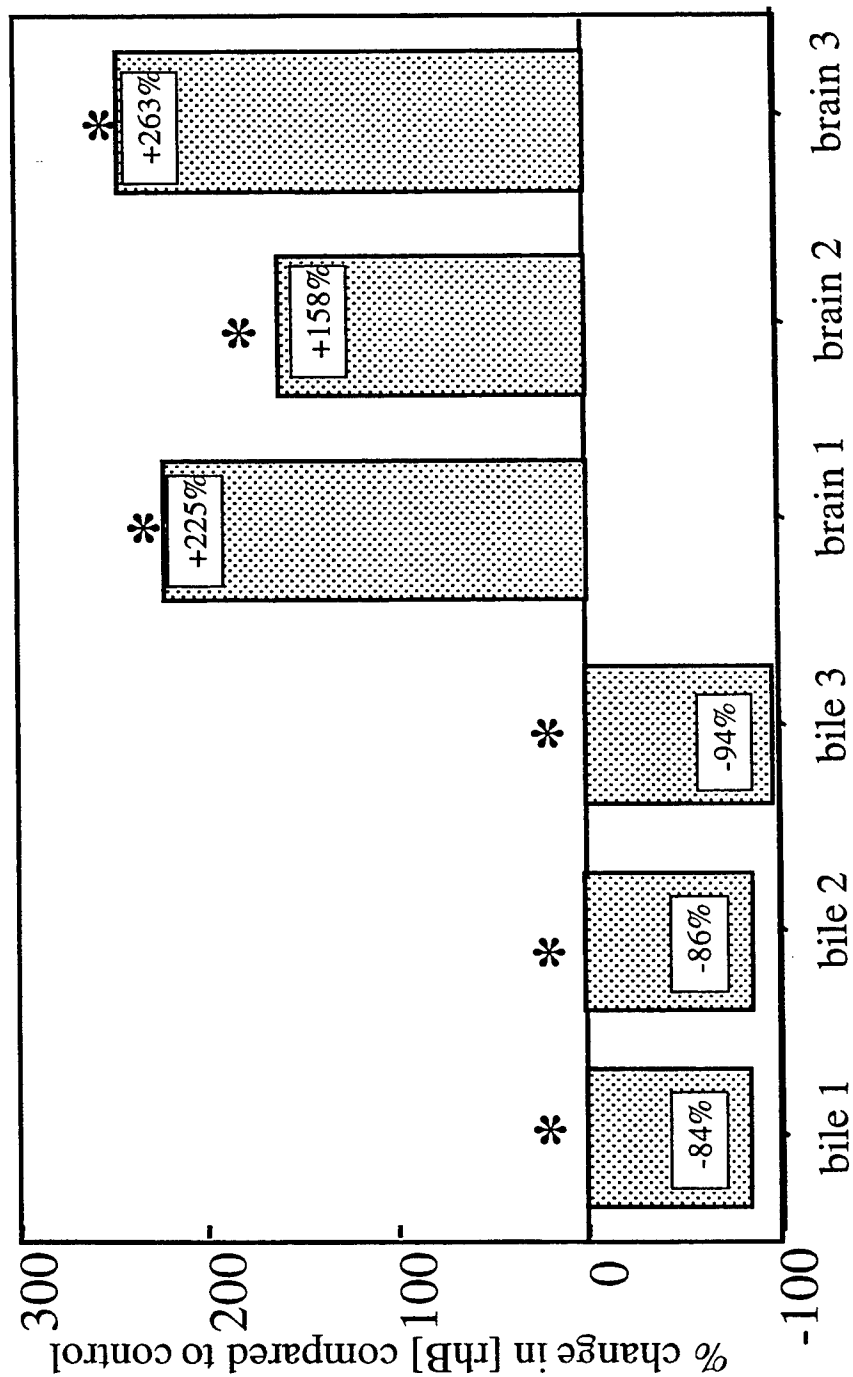
Figure 8. Percent change in rhodamine B concentration in organs of treated fish (0.3 μg/g rhB + 10 μg/g cyclosporin A) compared with control fish (0.3 μg/g rhB) in three experiments (Experiment 1: N=19 fish/treatment; Experiment 2 and 3: N=6 fish/treatment). No change in [rhB] in treated fish would would be indicated by a value of 0% while an increase would be > 0% and a decrease < 0%. * indicates that change in [rhB] in treated fish is significantly different from control fish (p≤0.05).

Table 1. Tissue-to-blood and bile-to-liver concentration ratios of rhodamine B in control versus cyclosporin A-treated fish.

	control	+CsA
brain-to-blood	0.12 ± 0.12	0.29 ± 0.28*
ovary-to-blood	0.96 ± 1.20	2.45 ± 2.89*
kidney-to-blood	3.15 ± 1.62	2.65 ± 1.49
gut-to-blood	2.28 ± 1.04	1.37 ± 1.17*
gill-to-blood	1.09 ± 0.56	0.87 ± 0.36
liver-to-blood	14.23 ± 4.39	10.37 ± 8.75
bile-to-liver	0.49 ± 0.40	0.14 ± 0.16*

Values are reported as mean ratio ± standard deviation. (*) indicates significant difference from control ($p \leq 0.05$).

Table 2. The effect of cyclosporin A treatment on rhodamine B accumulation in bile and brain

Experiment	Bile avg. [RhB] nmol/g	Bile avg % dose RhB %	Brain avg. [RhB] nmol/g	Brain avg % dose RhB %
1 control	4.00 ± 2.74	4.45 ± 4.15	0.09 ± 0.09	0.09 ± 0.09
+ CsA	0.70 ± 0.96*	0.72 ± 0.97*	0.28 ± 0.35*	0.28 ± 0.30*
2 control	5.01 ± 8.16	5.30 ± 8.38	0.12 ± 0.10	0.12 ± 0.08
+ CsA	0.72 ± 0.48*	0.91 ± 0.70*	0.31 ± 0.17*	0.31 ± 0.20*
3 control	4.49 ± 3.65	3.16 ± 3.41	0.19 ± 0.18	0.20 ± 0.17
+ CsA	0.28 ± 0.68*	0.13 ±0.31*	0.69 ± 0.35*	0.67 ± 0.30*

Average rhB concentration and percent of total dose measured in bile and brain of control fish (0.3 µg/g rhB) and +CsA treated fish (0.3 µg/g rhB + 10 µg/g CsA).
Sample size: Experiment 1 (N=19 fish/treatment); Experiment 2 and 3 (N=6 fish/treatment).
Values are reported as average ± standard deviation.
* indicates significant difference from control ($p \leq 0.05$).

Chapter 7

P-glycoprotein activity does not affect the *in vivo* distribution of ^{3}H-benzo[a]pyrene in a vertebrate model

(*Fundulus heteroclitus*).

Abstract

P-glycoproteins (P-gps), energy dependent efflux flippases, can prevent the cellular accumulation of a wide variety of moderately hydrophobic compounds. There are continuing questions concerning substrate specificity for environmental contaminants. Benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon (PAH), is a common environmental contaminant and known carcinogen that has been shown to induce P-gp in cell culture, and transport of B[a]P observed in several *in vitro* systems has been attributed to P-gp-mediated transport. We investigated whether P-gps are involved in B[a]P transport *in vivo* in a vertebrate model, the killifish (*Fundulus heteroclitus*), which is exposed to PAHs in its estuarine environment. This *in vivo* model for P-gp-mediated transport was previously established by determining that distribution of the fluorescent dye rhodamine B (rhB), a known P-gp substrate, was altered by the chemosensitizer drug (cyclosporin A (CsA)). The distribution of intramuscularly injected ^{3}H-B[a]P to bile, liver, brain or ovary was not affected by simultaneous intravenous injection of CsA. To evaluate further whether cytochrome P450 1A (CYP1A) metabolites of B[a]P may be transported by P-gp, we compared distribution of ^{3}H-B[a]P in fish previously treated

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with 2,3,7,8-tetrachlorodibenzofuran (TCDF), a model CYP1A inducer, to that in previously untreated fish. CsA did not affect the distribution of label in induced fish. Our results suggest that in contrast to rhB, the distribution of B[a]P and/or its metabolites generated *in vivo* are not influenced by P-gp-mediated transport in liver, brain, or ovary. We conclude that B[a]P should not be considered a P-gp substrate in *Fundulus heteroclitus* and likely other vertebrates.

Introduction

Transmembrane P-glycoproteins (P-gps) are responsible for certain multidrug resistance (MDR) phenotypes in tumor cell lines and in human patients (Juliano and Ling, 1976; Gerlach *et al.*, 1986). P-glycoproteins function as energy dependent efflux flippases that prevent the cellular accumulation of a wide variety of compounds. Known P-gp substrates are moderately hydrophobic, amphipathic (i.e. somewhat soluble in both lipid and water), low molecular weight, planar molecules with a basic nitrogen atom, cationic or neutral but never anionic; many of these substrates are natural products (Gottesman and Pastan, 1988; Endicott and Ling, 1989; Pearce *et al.*, 1990; Gottesman *et al.*, 1994). P-gp substrates also include endogenous compounds such as glucocorticoids (Naito *et al.*, 1989; Ueda *et al.*, 1992). Some environmental pollutants are also P-gp substrates (e.g. 7,12-dimethylbenz(a)anthracene, pentachlorophenol, and endosulfan)

(Phang et al., 1993; Cornwall et al., 1995; Bain and LeBlanc, 1996), but there is uncertainty regarding many common environmental contaminants.

Benzo[a]pyrene (B[a]P), a model polycyclic aromatic hydrocarbon (PAH), is a common environmental contaminant and known carcinogen (Buening et al., 1978), has been shown to induce P-gp in a nonparenchymal rat liver epithelial cell line (Fardel et al., 1996). Verapamil-sensitive active transport of B[a]P has been observed in both the adriamycin-resistant human breast cancer MCF-7 cell line which developed cross-resistance to B[a]P (Yeh et al., 1992), and in apical membrane vesicles (AMV) from normal human intestine (Penny and Campbell, 1994). This phenomenon has been attributed to P-gp-mediated transport (Yeh et al., 1992; Penny and Campbell, 1994). However, as discussed by Schuetz et al. (1998), more recent studies, including the discovery of additional classes of xenobiotic transporters, have drawn into question the in vitro evidence presented to support P-gp involvement in B[a]P transport. First, P-gp can be induced by toxicants that are not substrates, presumably as a cellular defense mechanism against the accumulation of deleterious metabolites that are P-gp substrates, such as ceramides that are produced during genotoxic and cytotoxic stress (van Helvoort et al., 1996; Thévenod et al., 2000). Thus the induction of P-gp by B[a]P in vitro does not conclusively indicate that B[a]P is a P-gp substrate. Second, in addition to P-gp, the multidrug resistance associated protein (MRP) (Cole et al., 1992; Krishnamachary and Center, 1993) and the lung resistant protein (LRP) (Kusakabe et al., 2000) are now known to be upregulated by adriamycin. Thus other transporters as well as P-gp might have been induced in the MCF-7 cell line derived against adriamycin. Third, the ability

of B[a]P to competitively inhibit azidopine labeling in MCF-7 cells (Yeh et al., 1992) is no longer believed to indicate a functional interaction with P-gp because azidopine is now known to bind to proteins other than P-gp (Scala et al., 1997). Finally, verapamil, which was demonstrated to inhibit B[a]P efflux in both MCF-7 cells and AMV, may also inhibit MRP and LRP (Wyler et al., 1997). Thus verapamil-sensitive transport of B[a]P in MCF-7 cells does not support P-gp as the sole transporter responsible for this phenomenon.

In light of this controversy, and the possibility that in vitro systems may not reveal accurately the role of P-gps in vivo, our goal was to investigate whether P-gps are involved in B[a]P transport in a vertebrate whole animal model, the killifish (Fundulus heteroclitus). Killifish express P-gp-like genes (Cooper, 1996) and elevated expression of hepatic P-gps (Cooper et al., 1999; Bard et al., 2000 in preparation-a) has been described in this species. Some populations of this fish also have developed resistance to exposure to PAHs in their estuarine environment (Williams, 1994; Prince and Cooper, 1995a; Prince and Cooper, 1995b; Hahn, 1998; Nacci et al., 1999; Elskus et al., 1999). We previously developed a rapid, inexpensive assay to simultaneously evaluate whether candidate xenobiotics are transported by P-gp in vivo in multiple organs of this species (Bard and Stegeman, 2000 in preparation).

Using a modified version of this assay, we evaluated whether in vivo inhibition of P-gp by a chemosensitizer drug (cyclosporin A (CsA)) altered the accumulation of radiolabeled ^{3}H-B[a]P in bile, liver, brain or ovary. The highly lipophilic structure of B[a]P is not typical of P-gp substrates. However, the parent compound B[a]P may be

oxidized through the action of the phase I enzyme cytochrome P450 1A to generate more hydrophilic compounds which are closer in physico-chemical properties to P-gp substrates. To evaluate whether CYP1A metabolites of B[a]P may be transported by P-gp, we also evaluated the distribution of total radioactivity in fish previously treated with 2,3,7,8-tetrachlorodibenzofuran (TCDF), a model CYP1A inducer, compared to untreated fish. Our results suggest that in contrast to our results with the known P-gp substrate rhB (Bard and Stegeman, 2000 in preparation), the distribution of B[a]P and/or its metabolites is not influenced by P-gp-mediated transport in liver, brain, or ovary.

Methods

Materials

Cyclosporin A and Rhodamine B (N-[9-(2-carboxyphenyl)-6-(diethylamino)-3H-xanthen-3-ylidene]-N-ethylethanaminium chloride) were purchased from Sigma (St. Louis, MO). Ringer's buffer consisted of 116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl2, 5 mM HEPES, pH 7.2.

^{3}H-Benzo[a]pyrene was purchased from Amersham (Piscataway, NJ). The tissue solubilizer Solvable™ was purchased from Packard Instruments (Downer's Grove, IL). 2,3,7,8-tetrachlorodibenzofuran was purchased from ULTRA Scientific (North Kingstown, RI).

Animal maintenance

Fundulus heteroclitus were collected in July, 1998 and July, 1999 from a putative clean field site (Scorton Creek, MA) 3-12 months prior to experiments. Previous chemical analysis has shown that PAHs are not detected in sediment from Scorton Creek (Moore *et al.*, 1995). Fish were maintained at Woods Hole Oceanographic Institution in flowing, filtered seawater at 15° C with aeration and thrice weekly were fed frozen brine shrimp and Tetramin flake fish food. Gravid females were selected for all experiments. Ovary/body weight ratios were 0.018 ± 0.016.

Rhodamine B $\pm$ cyclosporin A

As a positive control for P-gp-mediated transport, we present data from a previous study (Bard and Stegeman, 2000 in preparation). In that study, fish were injected via the caudal vein with 0.3 μg rhB dissolved in Ringer's buffer per g fish weight with or without 10 μg/g CsA suspended in Ringer's buffer (N=19/treatment). Fish were sacrificed by cervical scission one hour post-injection and liver, bile (the gall bladder was drained and discarded), brain, and ovary were removed and weighed. Each organ was homogenized in a buffer consisted of 50 mM Tris-HCl, 0.15 M KCl, pH 7.5. To provide sufficient sample volume for analysis, organs and bile were homogenized in the following ratios of organ weight to volume of homogenizing buffer: liver (1:10), bile (1:12), brain (1:12), and ovary (1:9). Samples were processed in a glass homogenizer with a drive powered tissue grinder. 100 μl of each homogenate were immediately loaded in triplicate onto a 96 well plate along with 100 μl of organ homogenates from untreated fish which were

loaded as "organ blanks" to account for native fluorescence of each organ. Rhodamine B fluorescence was measured by cytofluor fluorescence plate reader. Loaded plates were scanned once, then 10 pmol of rhB was added to each sample well but not to standard curve or organ blanks, and the plate was scanned a second time. The fluorescence due to rhB in each sample is equal to the measured fluorescence of the organ homogenate minus the fluorescence of the appropriate organ blank. The data were analyzed in three ways, as described previously (Bard and Stegeman, 2000 in preparation): percent of total dose of rhB in each organ; concentration of rhB in each organ; percent change in average rhB concentration in each organ in CsA-treated fish compared to fish given rhB alone.

2,3,7,8-Tetrachlorodibenzofuran treatment

2,3,7,8-Tetrachlorodibenzofuran was dissolved in corn oil at a concentration of 10 nmol/ml. Eighteen female fish were given intraperitoneal injections of 10 pmol TCDF per gram fish weight, a dose previously shown to induce ethoxyresorufin-O-deethylase activity (EROD), a measure of cytochrome P4501A function, by an order of magnitude in livers of teleost fish (Hahn and Stegeman, 1994) including *F. heteroclitus* (Bello, 1999). We have previously demonstrated that exposure to this dose of TCDF does not induce P-gp in killifish liver, brain, or ovary (Bard *et al.*, 2000 in preparation-a). Four days after dosing, TCDF-treated fish were exposed to ^{3}H-B[a]P with or without CsA as described below.

³H-Benzo[a]pyrene ± cyclosporin A

³H-Benzo[a]pyrene was dissolved in corn oil at a concentration of 7 µg/µl and specific activity of 1 µCi/mg. Fish were injected intramuscularly via the tail muscle with 28 µg ³H-B[a]P per gram fish weight. Then either CsA (10 µg/g body weight, suspended in Ringer's buffer) or only Ringer's buffer as a vehicle control was injected into the caudal vein. In order to determine if greater metabolism of B[a]P might influence the distribution of total radioactivity in various organs, we compared fish treated with TCDF to those fish treated with corn oil (N=18 fish treated with TCDF; N=16 corn oil fish). Fish were sacrificed three hours post-B[a]P-injection. This time point was selected based on results of a prior time course pilot study. In the pilot study the appearance of radiolabel in the brain, bile, and liver after an i.m. injection of the ³H-B[a]P was measured by scintillation counting at 2, 4, 9, and 12 hours post-injection (data not shown). We determined that sufficient quantities of the radiolabel would be detectable at 3 hours post-injection to permit us to undertake the experiment.

Approximately 1/3 of each liver was reserved for analysis of ethoxyresorufin-O-deethylase (EROD) activity. The remaining portion of liver, brain, and ovary were removed, weighed and placed in 10 ml glass scintillation vials. Bile and drained gallbladder were each weighed in separate scintillation vials. Liver, gallbladder, brain, and ovary were solubilized with Solvable™ according to the manufacturer's instructions except that a neutralization step to eliminate chemiluminescence, involving the addition of equal moles of HCl to Solvable™, was included before the liquid scintillation cocktail step. Samples were counted for tritium using a Beckman 5001TD Scintillation Counter

(Fullerton, CA). Count values for gallbladder and bile were combined for calculations

and are reported as bile in the results. The data were analyzed as both the concentration

and the percent of total dose of ^{3}H-B[a]P in each organ and bile.

Ethoxyresorufin-O-deethylase (EROD) activity assays

Approximately 1/3 of the liver from each ^{3}H-B[a]P-treated fish (both untreated

and TCDF-treated fish) was homogenized and centrifuged as previously described

(Stegeman *et al.*, 1979) to yield microsomes. The protein concentrations of microsomal

resuspensions were determined using the bicinchoninic acid (BCA) procedure (Smith *et*

al., 1985). Ethoxyresorufin-O-deethylase assays were performed using a cytofluor multi-

well plate reader as previously described (Hahn *et al.*, 1996).

Statistical Analyses

Differences between rhB accumulation (concentration and percent of total dose)

in each organ for fish treated with or without CsA were statistically analyzed by one-way

ANOVA using Fisher's protected LSD procedure using the SuperANOVA (Abacus

Concepts) statistical program; $p \leq 0.05$ was accepted as significant. Differences between

^{3}H-B[a]P accumulation (concentration and percent of total dose) in each organ in fish

treated with or without CsA and those fish which were previously untreated or which

were exposed to TCDF were statistically analyzed by one-way ANOVA using as

appropriate Fisher's Protected LSD or Dunnett's one-tailed procedure for unequal sample

sizes using the SuperANOVA (Abacus Concepts) statistical program; $p \leq 0.05$ was

accepted as significant. Linear regression analysis was performed between EROD activity (pmol/min/mg) in liver microcosms and ^{3}H-B[a]P concentration in each organ and bile using the same program.

Results

^{3}H-Benzo[a]pyrene ± cyclosporin A

In order to determine the effect of inhibiting P-gp on the bodily distribution of ^{3}H-B[a]P, fish were administered ^{3}H-B[a]P with or without the known P-gp inhibitor CsA. Three hours after the intramuscular injection sufficient levels of ^{3}H-B[a]P were present in bile, liver, brain and ovary to permit its detection by scintillation counter. The concentration of ^{3}H-B[a]P (Figure 2A) and the percent of total dose of ^{3}H-B[a]P detected in each organ of fish exposed to ^{3}H-B[a]P with or without CsA (Figure 2B) were calculated from the total radioactivity measured in each organ. For the purpose of this paper, the total radioactivity will be referred to as ^{3}H-B[a]P and will include both parent compound and any metabolites generated *in vivo*. The concentration (nmol/g tissue or μmol/L bile) of ^{3}H-B[a]P in organs of fish treated only with ^{3}H-B[a]P was greatest in bile, then decreasing in order liver > brain > ovary (Figure 2A). In these fish, the following proportions of injected ^{3}H-B[a]P were detected 3 hours post-intramuscular-injection: liver 0.225% of total dose; bile 0.167%; ovary 0.020%; and brain 0.018% (Figure 1B). The ^{3}H-B[a]P concentration ratio of bile/liver (3.3 ± 0.9) in ^{3}H-B[a]P plus CsA-treated fish did not significantly differ compared to fish only exposed to rhB (2.4 ± 1.0). The distribution of the ^{3}H-B[a]P was not altered in ^{3}H-B[a]P plus CsA treated fish compared

to fish given ^{3}H-B[a]P alone. We did not assess what portion of the radioactivity measured in the bile was parent compound compared with B[a]P metabolites. In order to determine if enhanced metabolism of ^{3}H-B[a]P influences the distribution of total radioactivity, we treated fish the cytochrome P450 1A model inducer TCDF.

TCDF treatment and EROD activity

Four days after fish were treated with TCDF, a cytochrome P4501A inducer, EROD activity in liver microsomes (1106 ± 749 pmol/min/mg) was >5 fold greater ($p < 0.05$) than EROD activity in microsomes from untreated fish (218 ± 187) (Figure 3). No significant differences in EROD activity in liver microsomes was detected either between untreated fish injected with only ^{3}H-B[a]P or ^{3}H-B[a]P plus CsA, or between TCDF-treated fish in the same comparison.

^{3}H-Benzo[a]pyrene ± cyclosporin A in TCDF-treated fish

In order to determine if enhanced metabolism of ^{3}H-B[a]P influences the distribution of total radioactivity in various organs, we compared its distribution in previously untreated fish to fish previously exposed to TCDF which had induced activity of cytochrome P450 1A, a phase I enzyme responsible for metabolism of B[a]P to more hydrophilic compounds (Figure 2). Fish with induced CYP1A should have a higher capacity to generate B[a]P metabolites compared to untreated fish. If moderately hydrophobic ^{3}H-B[a]P metabolites were transported by P-gp preferentially over the highly lipophilic ^{3}H-B[a]P parent compound, then one would expect, for example, to

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detect more total radioactivity in the bile of TCDF-treated fish exposed to ^{3}H-B[a]P compared to previously untreated fish. Furthermore, the addition of CsA in TCDF-treated fish would inhibit the transport of ^{3}H-B[a]P CYP1A metabolites leading to depressed total radioactivity in bile of these fish.

In our study, for fish previously exposed to TCDF, CsA treatment was not associated with statistically significant changes in the total radioactivity detected in bile or in any of the examined organs compared to that in TCDF-treated fish given ^{3}H-B[a]P alone. The ^{3}H-B[a]P concentration ratio of bile/liver did not significantly differ between ^{3}H-B[a]P plus CsA-treated fish (3.4 ± 2.9) compared to fish only exposed to ^{3}H-B[a]P (3.0 ± 1.9). We anticipated that there might be a greater amount of total radioactivity appearing in bile of TCDF -treated fish compared to untreated fish. However, the ^{3}H-B[a]P concentration ratio of bile/liver also did not differ between TCDF-treated fish and previously untreated fish. This lack of difference in concentration ratio suggests that, CsA did not affect the biliary secretion of ^{3}H-B[a]P in either TCDF or untreated fish. The distribution of total radioactivity in each organ did not significantly differ between previously untreated fish and TCDF-treated fish with elevated EROD. We do not know what portion of the radioactivity measured in the bile was parent compound compared with B[a]P metabolites.

EROD activity (pmol/min/mg) in liver microsomes of both previously untreated and TCDF-treated fish administered only ^{3}H-B[a]P was not correlated with the concentration of ^{3}H-B[a]P in bile (linear regression $r^2=0.035$, $p>0.47$) (Figure 4). EROD activity did not correlate with the concentration of ^{3}H-B[a]P in bile in all fish ($r^2<0.0001$).

No correlation (p>0.05) was detected between EROD of both previously untreated and TCDF-treated fish administered only ^{3}H-B[a]P and the concentration of ^{3}H-B[a]P in liver (y=-0.001x+12, r^2=0.017), ovary (y=-0.001x + 4, r^2=0.003) brain (y=0.0001x+1.4, r^2=0.053). Linear regressions between EROD activity and ^{3}H-B[a]P concentration or percent of total dose ^{3}H-B[a]P in each organ using data for all fish, or for previously untreated fish only, or for only TCDF-treated fish also showed no correlation (data not shown).

Rhodamine B ± cyclosporin A

As a positive control for P-gp-mediated transport, we examined the data for distribution of rhB, a known P-gp substrate, that we generated in a previous study (Bard and Stegeman, 2000 in preparation) in order to compare them with our ^{3}H-B[a]P disposition data. P-gp distribution is presented as the concentration of rhB (Figure 1A), the percent change in concentration (Figure 1A, inset), and the percent of total dose of rhB detected in each organ of fish exposed to rhB with or without CsA (Figure 1B).

Discussion

We previously characterized P-gp expression in a vertebrate model system, the killifish (*F. heteroclitus*) (Bard *et al.*, 2000 in preparation-a) and developed an assay to measure the *in vivo* P-gp-mediated transport of xenobiotics in this species (Bard and Stegeman, 2000 in preparation). Using a modified version of this assay, we evaluated whether the distribution of ^{3}H-B[a]P and its metabolites generated *in vivo* are influenced

by P-gp-mediated transport in killifish. As a positive control for P-gp-mediated transport, we also examined the data for distribution of rhB, a known P-gp substrate, that we generated in a previous study (Bard and Stegeman, 2000 in preparation) and compared them to our results.

In our previously reported study, the percent change in rhB concentration in organs of fish receiving rhB Compared to fish treated also with CsA (Figure 1A inset) showed that CsA-treated fish had dramatically ($p<0.05$) lower concentrations of rhB in bile (83% less than in bile of rhB only fish) and a slight though statistically significant decrease in rhB concentration in liver (29% less)(Bard and Stegeman, 2000 in preparation). In contrast, co-administration of CsA significantly increased the rhB concentrations in brain (225% greater than in rhB only fish) and ovary (226% greater) (Bard and Stegeman, 2000 in preparation).

If ^{3}H-B[a]P were transported by P-gp one would expect to observe similar trends as that seen in the rhB positive control, i.e. inhibition of P-gp by CsA is associated with lowered secretion of label into bile and increased accumulation in brain and ovary. However in this experiment, the distribution of the ^{3}H-B[a]P was not altered in ^{3}H-B[a]P plus CsA treated fish compared to fish given ^{3}H-B[a]P alone. In fish previously treated with the CYP1A inducer TCDF, we observed no difference in distribution of total radioactivity between fish injected with ^{3}H-B[a]P plus the P-gp inhibitor CsA versus those given ^{3}H-B[a]P alone. Furthermore, no difference in ^{3}H-B[a]P disposition was observed between TCDF-treated and untreated fish. We had anticipated that there would be greater radiolabel in the bile of induced fish compared to untreated fish due to the

enhanced metabolism of the highly lipophilic B[a]P by CYP1A to more hydrophilic metabolites which may be more easily excreted from the liver. In contrast to expectations, no difference in total radiolabel in bile was detected between induced and untreated fish. However, we did not analyze what proportion of radiolabel in bile consisted of B[a]P parent compound compared to CYP1A and other metabolites. In a previous study, the identity of radiolabelled constituents in the bile was evaluated in rainbow trout (*Oncorhynchus mykiss*) 48 hours after fish received an intra-arterial injection of ^{3}H-B[a]P (Seubert and Kennedy, 2000). Less than 2% of the radiolabel in bile consisted of parent compound, while >2% were phase I metabolites (mostly CYP1A metabolites), ~13% was the aqueous soluble fraction assumed to be glutathione conjugates, and the remaining biliary radioactivity consisted of approximately equal proportions of glucuronide, sulfate and unknown conjugates (Seubert and Kennedy, 2000). These results were consistent with other B[a]P pharmacokinetic studies in teleost fish including English sole and starry flounder (Varanasi *et al.*, 1986), gulf toadfish (Kennedy *et al.*, 1989), and the common carp (Steward *et al.*, 1991). Based on these studies, we can assume that in killifish the vast majority (~98%) of radiolabel in the bile probably consisted of B[a]P metabolites. The presence of a greater level of radioactivity in the bile (average ~35μmol/l among all fish given only B[a]P) compared to the liver (15 nmol/g among all fish given only B[a]P) indicates that 3 hours was sufficient time for fish to generate hepatic B[a]P metabolites which were then excreted into the gallbladder.

To explain why there was no difference in radiolabel detected in CYP1A induced fish versus uninduced fish, we compared of the amount of B[a]P in the liver (average

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~1.8 nmol) and in the bile (average ~2.6 nmol), to the microsomal EROD activity in TCDF treated fish (average ~1100 pmol/min/mg) and untreated fish (average ~200 pmol/min/mg). The amount of B[a]P reaching the liver within 3 hours was so small that even the ~5-fold lower level of CYP1A activity in uninduced fish would appear to be adequate to accommodate the metabolism of the B[a]P. Thus with such small amounts of B[a]P present in the hepato-biliary system we would not expect to see a difference in the distribution of radiolabel between CYP1A induced and uninduced fish. Difference in the distribution of radiolabel between CYP1A induced and uninduced fish might not be apparent until a later time point when a sufficiently large amount of B[a]P would be present in the liver that would challenge the capacity of the uninduced fish to metabolize the greater amount of accumulated B[a]P.

Our results suggest that neither B[a]P nor its metabolites are transported by P-gp *in vivo* in killifish. Our conclusion is in agreement with a recent study which showed no difference in B[a]P disposition between mdr1a knockout mice and wildtype mice (Schuetz *et al.*, 1998). That study is the only prior published report to assess P-gp involvement in B[a]P cellular retention in whole animals but it did not assess whether P-gp is involved in the transport of B[a]P metabolites. We agree with Schuetz and collaborators that prior observations of B[a]P active transport in *in vitro* systems were erroneously attributed to P-gp-mediated transport. Recent work in a P-gp negative KB-3-1 epidermoid pharyngeal carcinomas cell line suggests that the active transport of B[a]P in these cells may be facilitated by another class of transporters (Cheng *et al.*, 2000): LRP, the major vault protein localized to nuclear pore complexes, which may be involved

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in nucleocytoplasmic transport (Chugani *et al.*, 1993; Hamill and Suprenant, 1997). KB-3-1 cells which were selected for resistance to doxorubicin and B[a]P were P-gp negative and showed no elevated expression of MRP compared to parental cells, but showed a correlation between LRP expression and B[a]P resistance (Cheng *et al.*, 2000). In resistant cells, B[a]P accumulation was localized to the cytoplasm and little was detected in the nucleus, in contrast with parental cells which showed even distribution of B[a]P throughout the cytoplasm and nucleus (Cheng *et al.*, 2000). Further functional data is required characterizing B[a]P distribution when LRP function is inhibited in this and other cell models before LRP can be declared responsible for B[a]P transport previously reported *in vitro*. And additional work in whole animals would be needed to evaluate whether B[a]P transport *in vivo* is influenced by LRP or if as yet uncharacterized carriers are responsible. Determining whether B[a]P and its metabolites are translocated by the same or different transporters is also important for understanding the pharmacokinetics of this model PAH.

In characterizing the cytoprotective role of P-gp against accumulation of environmental xenobiotics, the valuable work being conducted in established *in vitro* systems and in mdr null mice, would be complemented by *in vivo* studies in natural vertebrate populations, such as killifish. The assay used in this study is an inexpensive and rapid method to assess whether a tested xenobiotic or its cytochrome P450 1A metabolites is transported by P-gp in several organs of a small fish which is easily maintained in large numbers in the laboratory. The use of this species permit analysis of large sample sizes for each treatment. Large sample sizes are especially important

because the magnitude P-gp expression in these fish reflects the large individual variability observed within wild populations (Bard *et al.*, 2000 in preparation-b) compared to inbred rodent strains.

The correct classification of contaminants as substrates for P-gp or other transporters is important for our understanding of the impact of anthropogenic pollutants, including 7,12-dimethylbenz(a)anthracene, pentachlorophenol, and endosulfan, on the ecology of the receiving environment and on the health of natural populations including humans. Our results suggest that P-gp activity does not influence exposure to B[a]P or its CYP1A metabolites in teleost fish which might be exposed to such contaminants in its natural habitat. We concur with Schuetz and colleagues that B[a]P should no longer be considered a P-gp substrate in vertebrates. The *in vivo* assay used here could be applied to assess the potential of other environmental contaminants, including additional PAHs, to be transported by P-gp.

Acknowledgments

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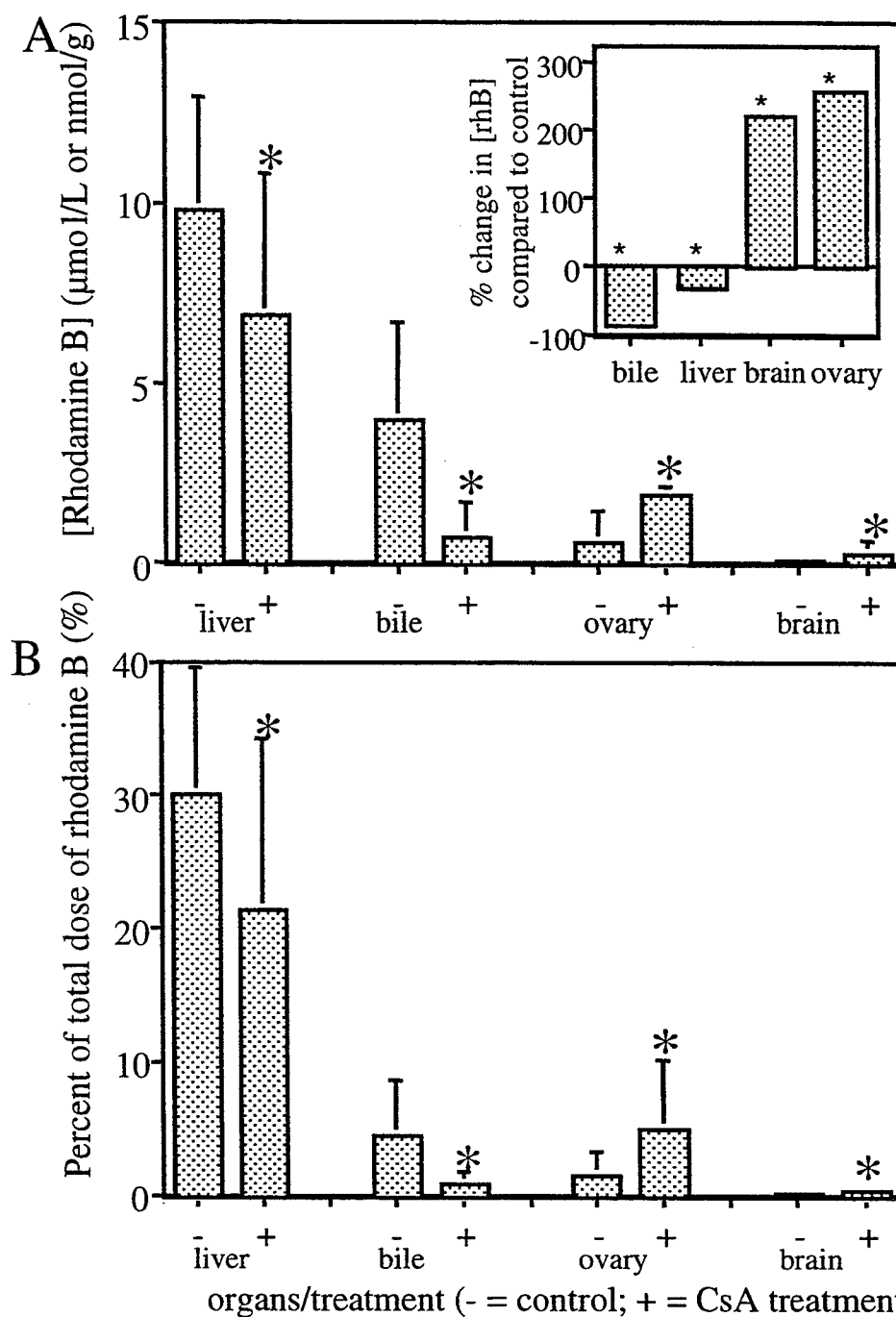
Figure 1. (A) Rhodamine B concentration and (inset) percent change in average rhodamine B concentration, and (B) percent of total dose of rhodamine B detected in organs of treated fish (0.3 μg rhB/g fish weight + 10 μg/g cyclosporin A) compared with control fish (0.3 μg/g rhB). Values are reported as (A) μmol/L for bile or nmol/g for organs and (B) percent of the original total dose of rhB ± standard deviation. * indicates significant difference from control (p≤0.05). For inset A, no change in average [rhB] in CsA-treated fish compared to control fish is indicated by a value of 0% while an increase is >0% and a decrease <0%. For inset A, * indicates that average [rhB] in organs of CsA-treated fish is significantly different from that of control fish (p≤0.05). Figure uses data generated during a previous study (Bard and Stegeman, 2000)

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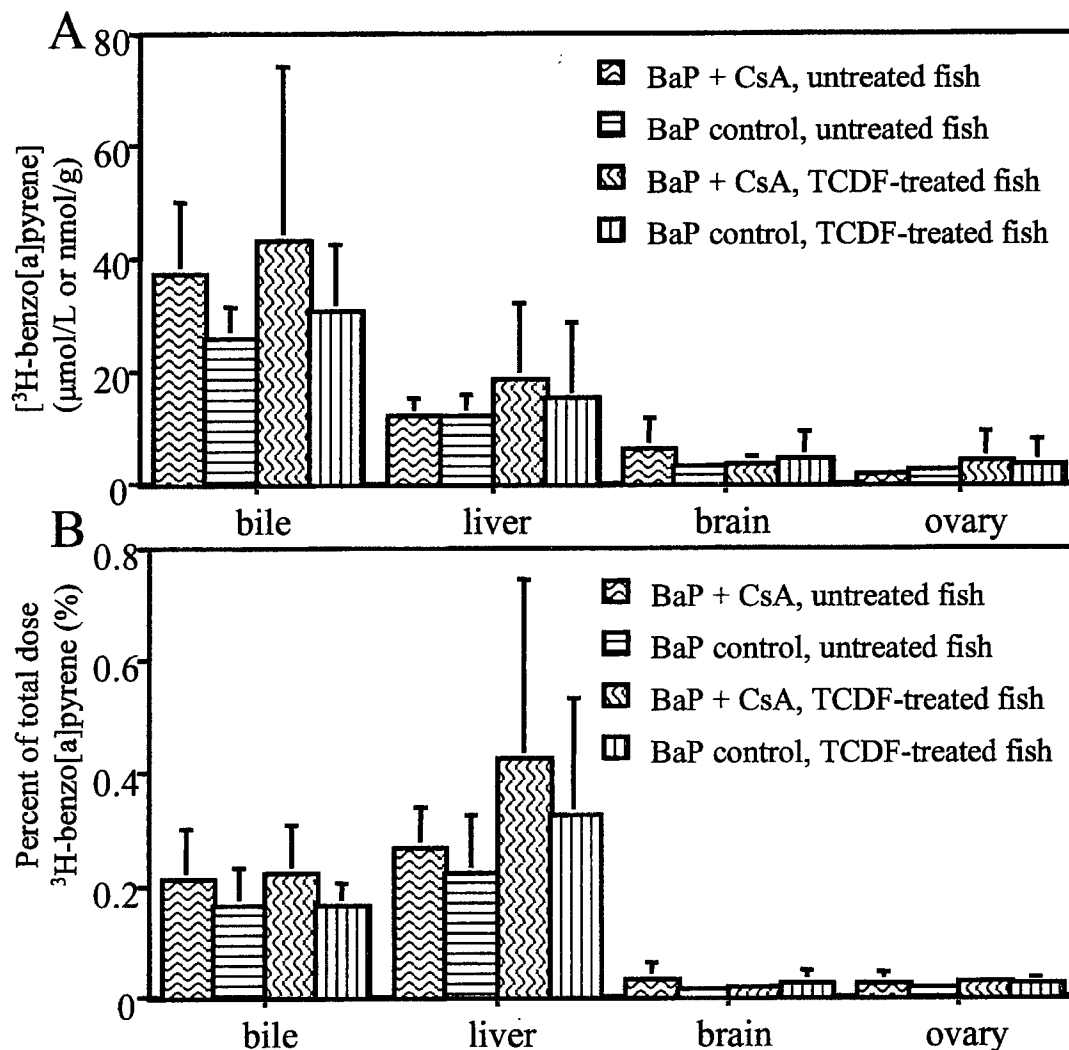
Figure 2. (A) ^{3}H-benzo[a]pyrene concentration and (B) percent of total dose of ^{3}H-BaP detected in organs of previously untreated and TCDF-treated fish exposed to 28 μg ^{3}H-BaP/g fish weight ± 10 μg/g cyclosporin A. Values are reported as (A) μmol/L for bile or nmol/g for organs and (B) percent of the original total dose of ^{3}H-BaP (%) ± standard deviation. No treatment yielded a value that was significantly different (p≤0.05) from previously untreated control fish exposed only to BaP.
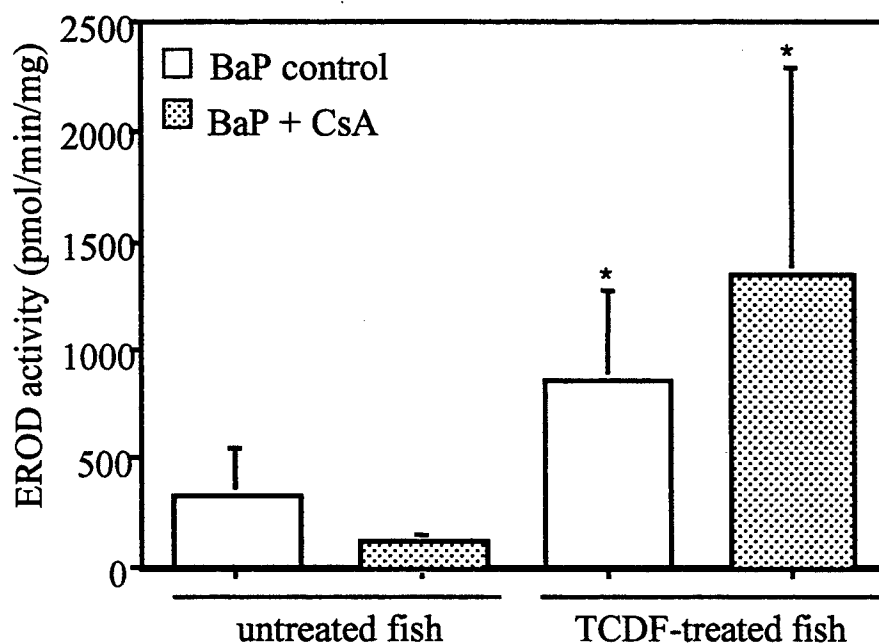
Figure 3. Ethoxyresorufin-O-deethylase activity in liver
microsomes of previously untreated fish and those treated with 10
pmol TCDF/g fish weight. Untreated and TCDF-treated fish
where further exposed to 28 μg ^{3}H-BaP/g fish weight ± 10 μg/g
cyclosporin A. Values are reported as pmol/min/mg EROD
activity ± standard deviation. * indicates significant difference
from previously untreated fish exposed to BaP control (p≤0.05).
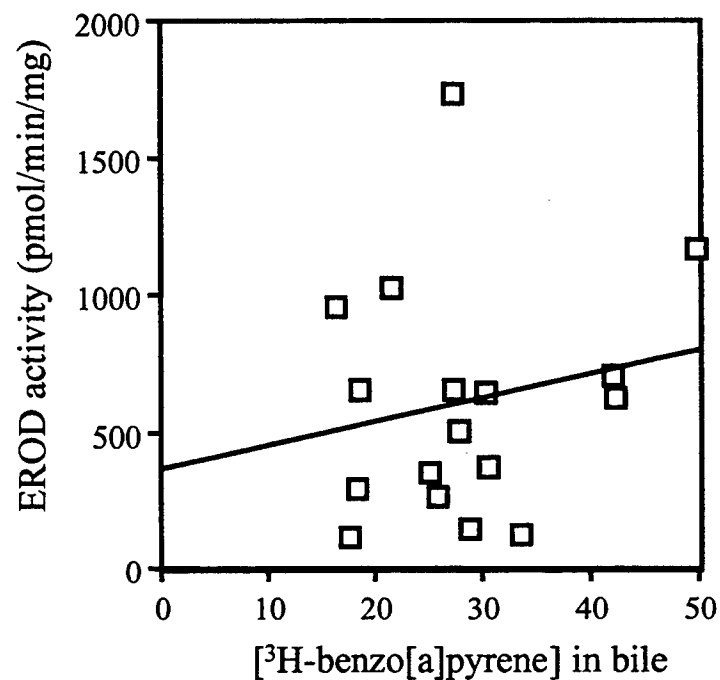
Figure 4. Ethoxyresorufin-O-deethylase activity (pmol/min/mg) in liver microsomes versus concentration of ^{3}H-benzo[a]pyrene in bile of both previously untreated and TCDF-treated fish which were exposed only to 28 μg ^{3}H-BaP/g fish weight ($y = 8.529x + 366.974$, $r^2 = 0.035$, $p>0.47$).

Chapter 8: Conclusions

The combined results of all the studies presented in this thesis demonstrate that P-glycoprotein (P-gp) activity is an important mechanism of multixenobiotic resistance for fish, which are commonly exposed to anthropogenic contaminants and naturally occurring toxins. We examined several questions: first, whether P-gp expression in cholangiocellular carcinomas resembles classical mdr; second, whether P-gp(s) in multiple organs of fish are induced by exposure to environmental xenobiotics; third whether P-gp is involved in the induction of CYP1A; and finally we developed an assay to investigate P-gp mediated transport of xenobiotics *in vivo*.

First, in our study of winter flounder from a highly contaminated site in Boston Harbor, Massachusetts, we discovered that the cholangiocellular carcinomas in these fish did not exhibit the classical multidrug resistance phenotype commonly observed in hepatocellular carcinomas in both fish and mammals. In contrast to expectations, we observed a general lack of P-gp expression in cholangiocellular carcinomas and associated vacuolated cells of winter flounder, while P-gp was highly expressed in bile canaliculi of non-tumorous liver parenchyma surrounding the cholangiocellular carcinoma. It would appear that the presence of hepatic disease in these fish may be responsible for increased P-gp expression in hepatic parenchyma. In our flounder specimens, elevated P-gp in the normal liver parenchyma surrounding tumors may be a hepatoprotective response to stimulate elimination of accumulated cytotoxic bile constituents in response to mild chronic cholestasis.

Second, we observed that P-gp was induced in blennies exposed to oil and in blennies freshly collected from the field compared to fish depurated in clean tanks. We investigated whether P-gps contribute to the xenobiotic resistant phenotype observed in a natural population of killifish exposed to planar halogenated aromatic hydrocarbons at the New Bedford Harbor Superfund site (NB). We had anticipated that if P-gp-mediated transport of xenobiotics contributes to the resistant phenotype observed in NB fish, then elevated P-gp expression would be detected in liver and intestine of these fish compared to fish from a reference site at Scorton Creek (SC). However in contrast to expectations, hepatic P-gp levels were lower in NB fish than SC fish. Consistent with our expectations, P-gp expression was detected in the intestinal epithelium of the majority of freshly collected NB fish compared to few SC fish. Elevated expression in different organs might also be due to tissue specific expression of the two fish P-gp isoforms, which we were unable to distinguish between in our experiment. Elevated intestinal P-gp isoform(s) in NB fish might provide a barrier against absorption of P-gp substrates/inducers and thus limit the amount of these compounds exported to the liver which might account for the lower hepatic P-gp isoform(s) levels in NB fish compared to SC fish. If this interpretation of the results is correct, then P-gp(s) may play a role in the resistance this population has acquired to the toxic effects of contaminants at the NB site. However, the lack of induction of P-gp in TCDF-treated fish and the fact that hepatic and intestinal P-gp levels did not further decrease after 8 days depuration, suggest that the highly lipophilic contaminants such as planar HAHs might not be responsible for P-gp induction. Rather the basis for differences in elevated levels of hepatic P-gp isoform(s)

(SC>NB) and intestinal P-gp isoform(s) (NB>SC) in freshly collected fish might reflect differing environmental exposure to moderately hydrophobic anthropogenic contaminants or microbial, algal, plant or other natural products via the sediment or diet at each site.

Third, we addressed the question of whether P-gp is involved in the induction of CYP1A in several studies. In our study of blennies, we found that the average hepatic P-gp and CYP1A expression was elevated in blennies exposed to multiple xenobiotics in the field or to oil in the laboratory compared to control animals, but induction occurred in different cellular locations. What compounds are acting to induce P-gp and CYP1A in these exposure experiments is unknown. In winter flounder, we had observed a lack of correlation between CYP1A and P-gp expression in the examined populations. Not all exposed individual fish had elevated expression of both hepatic P-gp and CYP1A. BNF, the model CYP1A inducer and aryl hydrocarbon receptor agonist did not induce P-gp in blennies. In killifish (*Fundulus heteroclitus*) injected with another model CYP1A inducer 2,3,7,8-tetrachlorodibenzofuran (TCDF) showed no induction of P-gp. These results suggest that P-gp is not regulated by the aryl hydrocarbon receptor pathway, although P-gp and CYP1A may both be induced in blennies under certain exposure regimes. Induction of P-gps may be due to exposure to CYP1A parent compounds or metabolites, or to cellular stress from cytotoxic or genotoxic agents. While our data indicate that CYP1A and P-gp are not coordinately regulated, these proteins may play complementary roles in cellular detoxification. .

Finally, we developed an inexpensive, simple, fast *in vivo* assay to measure P-gp activity in multiple organs in a vertebrate model species, killifish. In our experiments, the P-gp chemosensitizer cyclosporin A (CsA) dramatically decreased the biliary efflux of the model P-gp substrate rhodamine B (rhB) and strikingly increased accumulation in brain and ovary. These results are consistent with an expected inhibition of P-gp. These data suggest that in addition to hepatic transport, P-gp is an important mechanism of xenobiotics resistance in tissues such as the brain, ovary, and possibly developing oocytes that may be particularly sensitive to the accumulation of toxin compounds. We used a modified version of this assay to evaluate whether an environmental contaminant which induces P-gp, benzo[a]pyrene (B[a]P), is a true P-gp substrates. Our results suggest that P-gp activity does not influence exposure to B [a]P or its CYP1A metabolites in teleost fish which might be exposed to such contaminants in its natural habitat. We suggest that neither B[a]P nor its CYP1A metabolites are P-gp substrate in vertebrates.

The *in vivo* assay used here could be applied to assess the potential of other environmental contaminants to be transported by P-gp. The correct classification of contaminants as substrates for P-gp or other transporters is important for our understanding of the impact of anthropogenic pollutants on the ecology of the receiving environment and on the health of natural populations including humans. Understanding the effect of P-gp activity on xenobiotic disposition at the organismal level is important for evaluating, for example, health risk of environmental contaminants to a human population. The high individual variability of P-gp expression seen in natural populations of fish is more similar to the distribution of P-gp expression that one might

expect in a heterogeneous human population than in inbred rodent stocks. Fish which are continually challenged by exposure to water-borne anthropogenic contaminants and natural product toxins in their natural environment may be a good vertebrate model system to study the *in vivo* function of P-gp as a multixenobiotic resistance mechanism at the organismal and population levels.

Appendix

Global transport of anthropogenic contaminants and the consequences

for the Arctic marine environment

REPORTS

Global Transport of Anthropogenic Contaminants and the Consequences for the Arctic Marine Ecosystem

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Three major groups of anthropogenic contaminants are atmospherically transported thousands of kilometres from mid- and low-latitude sources to the Arctic troposphere: acidifying gases (SO$_x$), heavy metals, and persistent organic pollutants (POP). Sulphur aerosols may cause acid rain and promote climate change in the Arctic. Heavy metals and organic pollutants are adsorbed by plankton at the base of the food web and biomagnified to significant levels at higher trophic levels. Top Arctic predators such as seals, cetacea, and polar bears have surprisingly high levels of contamination. Northern Inuit communities rely on marine mammals for a large portion of their diet and are therefore vulnerable to contaminant exposure. This paper examines transport mechanisms, source of influx of the major pollutants, susceptibility of the Arctic food web to contamination, and the impact on the health of Northern indigenous communities. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Arctic; contaminant; pollutant; human health; marine environment.

The Arctic is generally considered to be one of the last pristine regions on Earth. The Arctic ecosystem is populated by few people, has minimal commercial fishing, little industrial activity, and is therefore imagined to be relatively unaffected by local human activity. These generalizations hold true for most of the Arctic except for highly industrialized regions of the Russian Arctic including the Kola Peninsula, Pechora and Ob/Yenisey river basins.

Growing evidence indicates, however, that there has been long-distance atmospheric transport of anthropogenic contaminants from mid- and low-latitude sources to the Arctic (Daisey *et al.*, 1981; Rahn, 1981; Barrie, 1986; Barrie and Bottenheim, 1991; Oehme, 1991; Pac-

yna, 1995; Oehme *et al.*, 1996). Three major pollutant groups are of growing concern: acidifying gases (SO$_x$) from Eurasian smelters and industry (Barrie *et al.*, 1989); heavy metals from fossil fuel combustion and mining (Akeredolu *et al.*, 1994); and persistent organic pollutants (POP) including pesticides used in agriculture (e.g. dichlorodiphenyltrichloroethane (DDT), toxaphene, hexachlorocyclohexane (HCH), and chlordane) and polychlorinated biphenyls (PCBs) leached from electronic transformers (Muir *et al.*, 1992). Contamination of Arctic air and subsequently seawater has led to bioaccumulation of pollutants by plankton (Bidleman *et al.*, 1989). Contaminants biomagnify through the Arctic marine food chain to levels in top predators, including humans, which may have adverse physiological effects (Dewailly *et al.*, 1989; Bacon *et al.*, 1992). Several excellent reviews on Arctic contamination have been previously published including a compilation of contaminant data for the marine ecosystem (Muir *et al.*, 1992); chlorinated hydrocarbon contamination in Arctic marine mammals (Norstrom and Muir, 1994; Tanabe *et al.*, 1994); chemical residues and biological response in contaminated Arctic aquatic animals (Lockhart, 1995); and the transport and biological effects of Arctic contaminants from global and local sources (MacDonald and Bewers, 1996). Recently, the results of 6 yr of research in the Canadian Arctic by the Northern Contaminants Program was published in the *Canadian Arctic Contaminant Assessment Report* (CACAR) (Government of Canada, 1997). The multinational Arctic Monitoring and Assessment Programme (AMAP) recently published the *AMAP Assessment Report: Arctic Pollution Issues* which includes monitoring data contributed from all eight circumpolar states: Canada, Denmark/Greenland, Finland, Iceland, Norway, The Russian Federation, Sweden, and the US of America (AMAP, 1998). This fully referenced, 800 + pp. report is currently the most comprehensive document available which details the status of the Arctic

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environment with respect to anthropogenic contaminants.[1] In addition to the topics covered by this review, the AMAP report describes physical, geographical, and ecological characteristics of the Arctic; contamination by radioactivity and petroleum hydrocarbons; climate change, ozone and ultraviolet radiation; anthropology of Arctic indigenous peoples; and international environmental policy recommendations (AMAP, 1998).

The present review will examine recent data on transport mechanisms, source of influx of the major pollutants, especially POPs, susceptibility of the Arctics marine food web to contamination, and impact on Northern human indigenous communities.

Transport Mechanisms

Air pollution is not confined to local areas and can be atmospherically transported tens of thousands of kilometres to remote regions such as the northern pole (Zoller *et al.*, 1973; Barrie, 1986; Barrie *et al.*, 1992). The winter phenomenon of "ice crystal haze" observed by K.R. Greenaway during 1940s surveillance flights and later coined "Arctic haze" (Mitchell, 1956) was discovered not to be wind-blown dust, but air pollution from the mid-latitudes (Kerr, 1979; Barrie and Bottenheim, 1991). Arctic haze is a mixture of aerosols containing acidifying SO_x and NO_x, coarse particles of soot, heavy metals (Iversen and Joranger, 1985), PAHs (polycyclic aromatic hydrocarbons) (Halsall *et al.*, 1997), and PCBs (Stern *et al.*, 1997). Refer to Barrie (1986), Barrie *et al.* (1992), Gregor (1991), Oehme (1991), Oehme *et al.* (1996) and AMAP (1998) for reviews of contaminant sources and pathways to the Arctic.

Volatile contaminants from mid- and low-latitudes reach the Arctic through a process known as "global distillation" (Goldberg, 1975). Many of the developing countries which still use environmentally persistent pesticides are located in the tropics. This region is characterized by elevated temperatures and heavy rainfall which promotes the rapid dissipation of contaminants through air and water (Tanabe *et al.*, 1994). Contaminants evaporate from soils in these warm regions and become available for atmospheric transport poleward where they condense out in the colder air (known as the "cold finger" (Ottar, 1981) or "cold condensation" effect (Wania and Mackay, 1993)). The adsorption of high molecular weight organic vapors to atmospheric particulate matter is enhanced by low temperatures (Bidlemen *et al.*, 1989). The theory of "global fractionation" describes how the most volatile contaminants, such as PCBs and HCH, travel to the highest latitudes, while less volatile compounds, such as

DDT, are less readily distilled and tend to remain near their source region (Simonich and Hites, 1995). Thus the volatile contaminants are apt to condense in the coldest Northern region and since this area is a small proportion of the Earth's surface, these contaminants can concentrate to surprisingly high levels in the Arctic.

Contaminants can be transported by *one-hop* or *multi-hop* pathways. *One-hop* describes the transport of a contaminant which enters the atmosphere in the source region, is directly transported to the Arctic and is deposited to the Earth's surface without returning to the atmosphere. Contaminants transported by *one-hop* pathways include involative organochlorines, e.g. DDT; less volatile PAHs, e.g. benzo[a]pyrene; acids; and heavy metals with the exception of mercury (Barrie *et al.*, 1997). In contrast, *multi-hop* contaminants undertake multiple atmospheric hops. Such chemicals include mercury and the more volatile organochlorines and PAHs (Barrie *et al.*, 1997).

Arctic haze is concentrated in the lower troposphere (up to 3 km altitude) and is most pronounced during the coldest months of the year from December–April (Pacyna, 1995). The seasonal cycle of anthropogenic emissions (e.g. April–June agricultural pesticide application) cannot adequately explain the distinct annual cycle of Arctic air pollution (Chernyak *et al.*, 1996). Iversen and Joranger (1985) proposed that a quasi-stationary, large-scale, meteorological phenomenon known as "blocking" is responsible for the seasonality of the poleward transport of pollutants. Episodically, mid-latitude source areas undergo periods of atmospheric stagnation due to the blocking of the westerlies by anticyclones (Dastoor and Pudykiewicz, 1996). The resultant stagnant weather conditions reduce contaminant scavenging potential and thus permit accumulation of pollutants over the source area. If a cyclonic system approaches the blocking high, a strong pressure gradient builds and forces a northward "surge" of contaminated air. The transport path may persist long enough to permit the pollutants to be swept into the Arctic troposphere (Raatz and Shaw, 1984; Weller and Schrems, 1996). Atmospheric transport rates were measured using vanadium (a non-crustal byproduct of heavy residual oil combustion) and manganese (derived from coal combustion) as chemical tracers because there are no significant sources of these elements in the Arctic. Investigators found that the tracer aerosols were transported from source to pole on the order of 7–10 days (Raatz and Shaw, 1984). Other studies have demonstrated that polluted air masses can reach the Arctic within 48–72 h (Oehme, 1991).

The occurrence of blocking highs corresponds well to observed seasonal variations of Arctic air pollution (Iversen and Joranger, 1985). Rapid changes in circulation systems are responsible for the episodic nature of Arctic aerosol pollution (Lejenäs and Holmén, 1996). The geographic position of blocking highs seasonally favours different sources. Eurasian sources, which are

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more available to the Arctic than North American emission sources, accounts for >50% of Arctic air pollution (Barrie, 1986). During winter, the strong Siberian anticyclone drives air from central Eurasia into the Arctic which then moves either over North America or into major cyclonic regions in the Aleutians and near southern Greenland (Barrie *et al.*, 1992). During the spring, the Siberian high pressure cell dissipates and western Eurasia makes the greatest contaminant contribution to the Arctic. During the summer, there is a weak north-to-south transport alternating with input from the north Pacific and north Atlantic (Barrie *et al.*, 1992). This sporadic summer transport originates from Europe > former USSR > nominal North American contribution (Raatz and Shaw, 1984). Pollution sources can be *fingerprinted* by analyzing snow for the ratio of (particulate residue)/(particulate residue + dissolved residue) (De Caritat *et al.*, 1998). These ratios allow distinction of different industrial processes which formed the contaminants and thus can distinguish between pollution sources, for example, ore smelting (water soluble deposition of Cu and S predominate) versus ore roasting (substantial particulate deposition of Cu and S) (De Cartitat *et al.*, 1998).

Once contaminants reach the Arctic, their lifetime in the troposphere depends on local removal processes. Pollutant removal process are inefficient in the Arctic especially during the winter due to low temperature, low solar radiation input, and low precipitation, thus low scavenging by wet deposition (Barrie, 1986; Weller and Schrems, 1996). As a result, contaminants which would be quickly photodegraded in warmer climes may persist in the Arctic. Eventually aerosol pollutants enter the terrestrial, aquatic and marine environments by gas exchange across the air-seawater interface or deposition of particles with adsorbed pollutants (Iwata *et al.*, 1993). Detection of tropical pesticides and other contaminants in remote Canadian and Alaskan Arctic freshwater lake and aquatic animals supports the atmospheric transport theory (Lockhart *et al.*, 1992; Wilson *et al.*, 1995).

The Arctic Ocean is surrounded by land mass and communicates with the southern oceans only via narrow passageways. Sea water enters the Arctic Ocean predominantly from the North Atlantic via the Barents Sea and the Fram Strait with a smaller contribution (approx. 17% of total input) from the North Pacific via the Bering Strait (Barrie *et al.*, 1997). Sea water transport from contaminant source to the Northern pole is unlikely to be a major factor in current Arctic contamination for most chemicals synthesized within the last few decades because ocean circulation from industrialized, temperate, coastal source regions is on the order of years to decades versus days for atmospheric transport (Schlosser *et al.*, 1995; Dastoor and Pudykiewiz, 1996). However, contaminants which are persistent, soluble, and conservative (i.e. unlikely to be scavenged by particles) may reach the Arctic by a combination of atmospheric and ocean transport (e.g. POP and HCH).

HCHs may be atmospherically deposited south of the Arctic into northward flowing sea currents which enter the Arctic Ocean predominantly through the Bering Strait (MacDonald and Bewers, 1996). Seventy-six percent of HCH input to the Arctic is by ocean currents, 22% by atmospheric deposition and 2% by rivers (Barrie *et al.*, 1997). Two-thirds of HCHs are subsequently transported from the Arctic by outflow through the Canadian Archipelago and the East Greenland Current (Barrie *et al.*, 1997). Currently, there is net removal of the α-HCH isomer from the Arctic presumably due to the decline in its use (Barrie *et al.*, 1997).

The cold Arctic ocean has been proposed to be a final sink for many contaminants (Iwata et al., 1993). Water "drains" from the Arctic "sink" into the North Atlantic, the Bering Sea and the Norwegian Sea (Doubleday, 1996). Potentially high levels of contaminants may be trapped in a water parcel which slowly travels over the deep sea, exposing benthic organisms to anthropogenic pollutants. The consequences of such contamination are unknown.

Major Contaminants

The following sections outline the three major contaminant groups of acidifying gases, heavy metals, and POP and describe the consequences for the Arctic ecosystem, marine organisms, and human health. Contaminants from mid- and low-latitudes have been detected in Arctic air and snow (Barrie *et al.*, 1992); aquatic organisms and freshwater (Verta *et al.*, 1990; Lockhart *et al.*, 1992; Wilson *et al.*, 1995); seawater, fog, and ice (Chernyak *et al.*, 1996); aquatic sediment, soil (Nriagu and Pacyna, 1988); plants (Simonich and Hites, 1995), the terrestrial food chain (Thomas *et al.*, 1992) and marine organisms (Holden, 1970; Addison and Smith, 1974; Wong, 1985; Jaworowski, 1989; Muir *et al.*, 1992; Norstrom and Muir, 1994). The bioaccumulative nature of POPs and the consequences for human Arctic populations which rely on marinelife for sustenance warrants special attention and will be examined in depth.

Acidifying Gases

Sources of acidifying gases

Both NO_x and SO_x are acidifying gases, but for this discussion only the most important gas for Arctic air mass contamination, the latter, shall be considered. Sulphur dioxide gas and sulphate, its oxidative product, are produced by fossil fuel consumption and the smelting of sulphide ores. Glacial records indicate that Arctic sulphate pollution has increased 70% since the turn of the century (Herron, 1982), a trend paralleled by global anthropogenic emissions (Pacyna, 1995). Sulphur dioxide and sulphate are atmospherically transported to the Arctic from mid-latitude sources by the previously described phenomenon of blocking. The total annual flux

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of sulphur into the Arctic is 2.3 Mt of which 2/3 is comprised of sulphur dioxide gas and 1/3 is sulphate (Barrie *et al.*, 1989). Eurasian emissions contribute more to the Arctic atmospheric sulphur load (94%) than North American (6%), because the latter sources are located further south of the westerlies and thus are meteorologically unfavoured for transport (Barrie *et al.*, 1989). The former sources are favoured for strong transport to the Arctic particularly in winter by the presence of the climatologically persistent Siberian high pressure system (Barrie, 1986). In addition to sources outside of the Arctic, acidifying gases are produced locally by smelters in Norilsk in Siberia, the Kola Peninsula, and in northern Scandinavia (AMAP, 1998).

Sulphur dioxide is a dominant component of Arctic gaseous air pollution (Iversen and Joranger, 1985) while sulphate is a major constituent of Arctic haze particles (Barrie, 1986). Atmospheric sulphate levels are distinctly seasonal, ranging from an average winter maximum of 56 $\mu g/m^3$ to a summer minimum of 14 $\mu g/m^3$ (Dastoor and Pudykiewicz, 1996). Yearly atmospheric sulphate averages much higher at Arctic air quality monitoring stations neighbouring smelters: Norilsk (140–160 $\mu g/m^3$), Sevettijärvi, northeastern Finland (400 $\mu g/m^3$), and Viksjøfjell, northern Norway in proximity to the Cu–Ni smelters on the Kola Peninsula (> 1000 $\mu g/m^3$) (AMAP, 1998). The lack of winter sunlight suppresses the irreversible transformation of sulphur dioxide to acidic sulphate. However, the abrupt change from complete darkness to light over a period of weeks in early spring precipitates a massive oxidation event which increases sulphate particulate levels. As a result, an Arctic haze maximum is apparent in March and April. During the summer, the higher precipitation rates contribute to wet deposition of sulphur to the Arctic ecosystem and an atmospheric minimum during this season (Barrie and Bottenheim, 1991).

Effect of acidifying gases on the Arctic ecosystem and human health

Two major effects of atmospheric sulphur contamination on the Arctic environment have been proposed: acidification of the sensitive Arctic ecosystem and perturbation of the solar radiation budget (Valero *et al.*, 1984). Wet deposition in the Arctic is slightly acidic, pH 4.9–5.2, due mainly to sulphate content. These levels are 5–10 times less severe than those measured for European and North American regions afflicted with environmentally destructive acid rain (Pacyna, 1995). However, selective leaching of hydrogen ions from snowmelt at the onset of spring can cause a 3–5-fold increase in acid concentration (Johannessen and Henriksen, 1978; Jeffries *et al.*, 1979). Such an acid shock may be potentially detrimental to organisms highly exposed to run off. Day-or week-long acid pulses at the onset of snowmelt in the Swedish sub-Arctic are believed to have caused the decline of fauna in small streams which normally have a pH above 6 (AMAP, 1998). Similar phenomenon

have been described in rivers in the Kola Peninsula and in eastern Finnmark, Norway close to the Russian border (AMAP, 1998). Lakes in Northern Norway have been acidified by SO_2 emissions from Russian smelters on nearby Kola Peninsula. The acidification has driven three Arctic char (*Salvelinus alpinus*) populations to extinction while 3 other populations of Arctic char and 8 populations of brown trout (*Salmo trutta*) are reduced (Hesthagen *et al.*, 1998). Extensive terrestrial vegetation damage from acidification has been observed in the area surrounding the Norilsk smelter and near the Kola Peninsula (AMAP, 1998). In acidified lakes and rivers neighbouring Arctic smelters, species diversity has declined due to the disappearance of acid-sensitive phytoplankton, invertebrates and fish (AMAP, 1998). Currently there are no studies which report any direct affects of acidifying gases on mammals or on the health of humans who reside in the Arctic (AMAP, 1998).

High levels of sulphate particulate aerosol also may increase summer albedo (percentage of incoming radiation reflected by the surface) and backscattering of short-wave radiation, favouring an atmospheric cooling (Dastoor and Pudykiewicz, 1996). However, cooling may be balanced by the effects of airborn soot which favour atmospheric warming (Blanchet, 1991). Climate is influenced by many different factors and is exceedingly difficult to model and predict. Historical climate changes have led to disruption of ecosystems via species emigration, immigration, and extinction. Dramatic climate changes precipitated by air pollution from outside the Arctic could potentially perturb the resident organisms. Major changes in species abundance and diversity at one trophic level may have repercussions at other trophic levels. The Arctic ecosystem, characterized by low diversity and a simple food chain, may be particularly susceptible to disturbance (Norstrom *et al.*, 1988). Refer to the AMAP report for a detailed discussion of Arctic climate change (1998).

Heavy Metals

Sources of heavy metals

Anthropogenic trace metals are predominantly transported from Eurasian industrial emission sites to the Arctic where they form a significant component of particulate pollution. Akeredolu *et al.* used a chemical transport model to calculate the total annual flux of antimony (4 t), cadmium (47 t), arsenic (285 t), vanadium (474 t), zinc (2350 t), and lead (2400 t) from Eurasia to the northern pole (1994). This flux represents 2–6% of the total source emissions. Eastern Europe contributed the greatest emissions for all metals except lead, which had equal contributions from the former USSR, western and eastern Europe. As with sulphur, a late winter maximum of trace metal Arctic air pollution was observed. Trace metal particles can contaminate the Arctic ecosystem via wet scavenging and dry deposition. Estimates of 11–14% of trace metals transported to the

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Arctic air mass are deposited in the polar region. Deposition rates are twice as efficient for trace metals as for sulphur (Akeredolu et al., 1994). The AMAP report contains extensive tables which detail the concentration of heavy metals in soils, sediment, wetlands, vegetation, birds, invertebrates, fish, and marine mammals at numerous sites around the Arctic (1998).

Effects of heavy metals on the Arctic ecosystem and human health

Organisms that consume particles with adsorbed trace metals can accumulate those metals in their body tissues. Some heavy metals, such as mercury and cadmium, can biomagnified up the food chain to detectable amount in top consumers (Hansen and Danscher, 1995; Caurant et al., 1996; Skaare, 1996). Exposure to heavy metals, such as mercury, arsenic, lead, and cadmium, can be detrimental to organisms. Most Arctic heavy metal contaminant work, and that which will be highlighted here, has focussed on mercury and to a lesser extent cadmium, selenium and lead. These heavy metals have been detected in the body tissues of Arctic organisms from invertebrates to marine mammals to Arctic humans (Dewailly et al., 1989; Wagemann, 1995; Dietz et al., 1996; Skaare, 1996; Wagemann et al., 1996). Contaminated marine mammals including ringed seals (*Phoca hispida*), beluga (*Delphinapterus leucas*) and narwhal (*Monodon monoceros*) are commonly eaten by the Inuit and other Arctic indigenous peoples (Chan et al., 1995). Dietary exposure is the major factor responsible for elevated heavy metal levels in Arctic human populations with the exception of cadmium. In Arctic populations, blood cadmium levels are closely associated with cigarette smoking and are independent of marine mammal consumption by non-smokers (Rey et al., 1997). Among Greenlanders, 80% of the adult population smokes and non-smokers are exposed to second hand smoke in the crowded dwellings (Bjerregaard, 1995). Blood cadmium levels due to tobacco exposure are elevated enough to warrant concern. The following sections outline heavy metal contamination in the Arctic ecosystem and the consequences for Arctic human health.

Mercury has both local natural geological sources in the Arctic as well as influx from combustion of coal and municipal garbage at low-latitudes. Due to geographically different geological conditions, pre-industrial mercury flux in Arctic and sub-Arctic lakes ranges from 0.7 to 54.35 $\mu g/m^2/yr$ (Landers et al., 1998). Mercury is susceptible to global transport due to high volatility and a 1 yr atmospheric half-life (Slemr and Langer, 1992). The concentration of coal combustion sources in the North American mid-west combined with atmospheric conditions creates a mercury corridor. Mercury is atmospherically transported in a northwesterly direction from sources in the mid-west to a high depositional zone north of Hudson Bay (Landers et al., 1998). Mercury concentrations in marine biota from the western Arctic

exceed those from the eastern Arctic due to geological phenomenon (Wagemann et al., 1996). Studies of northern lake sediments suggest that in all regions mercury levels increased little from 1500 to 1750 AD but then increased more rapidly from 1750, the onset of the Industrial Revolution, to today (Lockhart et al., 1993). The pre-Industrial levels indicate the background levels of natural mercury from mineral leaching while the post-Industrial increase presumably reflects the anthropogenic contribution. An examination of Pb^{210} dated lake sediment cores from an Arctic lake in Imitavik with no local source of contamination found atmospheric input of mercury began in the mid-18th century. Atmospheric deposition has increased about 6-fold during the past 240 yr in Imitavik (Hermanson, 1998). This increased mercury load is reflected in Arctic organisms as well.

Archaeological studies of burial sites in Greenland have shown that 500 yr-old seal hair contained much less mercury than modern seal hair (0.6 $\mu g/g$ versus 2.6 $\mu g/g$). Past and present Inuit Greenlanders depend on marine mammals for a large portion of their diet and may bioaccumulate contaminants to levels higher than that in their prey. As expected, a similar trend was seen in mercury levels in human hair samples from the previous era compared to contemporary Greenlanders (3.1 $\mu g/g$ versus 9.8 $\mu g/g$) (Hart Hansen et al., 1991). This increase is interpreted to be due to anthropogenic input of mercury from distal sources.

Relatively recent temporal trend studies compared liver mercury levels in Arctic beluga sampled in 1981–1984 to those sampled in 1993–1994 (Wagemann et al., 1996). In belugas which were $\geqslant 8$ yr old, mercury accumulated in more recent time at twice the rate of beluga sampled in the 1980s (Wagemann et al., 1996). Ringed seal liver sampled in 1987–1993 accumulated mercury at a rate 3 times higher than specimens collected 15–20 yr earlier (Wagemann et al., 1996; Muir et al., 1997).

Compared to marinelife in other oceans, mercury concentration is low in Arctic organisms (Joiris et al., 1997). The principle determinant of mercury concentration in marine biota is primary production. For a given mercury level in water, the lower the particulate matter, i.e. phytoplankton concentration, the greater the mercury concentration in the phytoplankters (Joiris et al., 1995). This "biomass effect" explains how bioaccumulation of mercury and other contaminants by phytoplankton and on up the food chain can occur more efficiently than predicted in less contaminated areas with low productivity, such as the Arctic (Delbeke and Joiris, 1988; Joiris and Overloop, 1991).

Dietz et al. report baseline concentrations of mercury (Hg), cadmium (Cd), lead (Pb), and selenium (Se) for Greenland marine organisms, from invertebrates to marine mammals, compiled over 20 yr (1996). Mercury, cadmium and selenium body burdens are high and correlate with age of animal while lead levels are low and no age correlation is observed. Mercury and cad-

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mium were found to increase at higher trophic levels. Mercury levels in the livers of marine organisms are ranked fish (0.005–0.569 µg/g) < seabirds ((0.046–2.67 µg/g) < seals, whales, polar bears (<21.6 µg/g). Cadmium concentrations in liver increase from fish (0.034–2.11 µg/g) < crustaceans and polar bears (0.477–7.79 µg/g) < birds, whales, seals (0.853–36.6 µg/g). Polar bears have relatively low Cd levels because Cd levels in their major food source, seal blubber, are very low.

Cadmium levels in kidneys of some Northwest Greenland ringed seals exceed the critical concentration of 200 ug/g wet weight (wet wt.), the threshold associated with kidney damage in mammals including humans (Dietz et al., 1998; WHO, 1992). A high incidence of renal tubular proteinurea would be expected at this level. However, even the most highly contaminated seal kidney cortex (726 µg/g Cd wet wt.) did not differ in renal morphology from uncontaminated seals (Dietz et al., 1998). This preliminary work suggests that ringed seals may be habituated to high cadmium levels without suffering renal damage and further investigation is neaded to elucidate tolerance adaptations.

Interpretation of contaminant data in Arctic birds is more complicated due to southern migration. Thick-billed Murres (Uria iomvia), Northern Fulmars (Fulmaris glacialis) and Black-legged Kittiwakes (Rissa tridactyla) breed in the Canadian Arctic and have been the subject of a contaminant monitoring study since the mid-1970's when pollutant residues were first detected (Braune, 1993, 1994; Braune and Gaston, 1997). Although much of the bird's contaminant body burden is accumulated at southern overwintering sites, a comparison of contaminant levels in eggs to chick body burdens demonstrated that all three species accumulate mercury, selenium and cadmium from the local Arctic environment during the summer.

A large percentage of Greenland marine mammals and seabirds had mercury and cadmium concentrations which exceed the conservative Danish food standard limits: 0.05 µg/g Hg in muscle; 0.1 µg/g Hg in liver and kidney; 0.05 µg/g Cd fish muscle, 0.5 µg/g Cd in mammals and birds, 0.5 µg/g Cd in liver, 1.0 µg/g Cd in kidney. Although the concentration of metals in Arctic wildlife is generally lower than in southern animals, the dependence of Northerners on a protein-rich country diet makes them susceptible to contamination. Inuits consume traditional food at twice the rate that the average Canadian consumes meat and fish (Wong, 1985; Gilman et al., 1997).

Health and Welfare Canada prohibits the consumption of marine organisms that exceed a relatively higher mercury limit of 0.5 µg/g (wet wt.). Mercury levels in belugas muscle, muktuk (raw skin and fat, an Inuit delicacy), liver, and kidney exceeded the Consumption Guideline substantially (Wagemann, 1995; Wagemann et al., 1996). Mean lead concentrations were low while zinc was concentrated 3 times higher in the skin than in other organs. Mercury levels increase progressively in

beluga skin layers with the highest concentrations in the outermost layer (1.5 µg/g wet wt.). During annual moulting, the top and underlying layer of skin is shed, thus reducing the total mercury skin tissue concentration by 20%. Consumers can reduce their heavy metal exposure by peeling off the most contaminated layer before eating muktuk.

Mercury loads in narwhal also exceeded the consumptive limit: muktuk, 0.59 µg/g wet wt.; muscle, 1.03 µg/g wet wt.; liver, 10.8 µg/g wet wt.; and kidney, 1.93 µg/g wet wt. (Wagemann et al., 1996). Ringed seal muscle was below the 0.5 µg/g threshold (0.39–0.41 µg/g wet wt.) but liver levels were 80 times higher. In ringed seals sampled from 1987 to 1993, mercury accumulated in the liver to levels 3 times higher than seals sampled 15–20 yr previously. Although beluga and narwhal livers are not eaten by the Inuit, ringed seal liver is considered a delicacy

Marine organisms appear to have the ability to concentrate mercury and other heavy metals without apparent toxic consequences (Caurant et al., 1996). Scientists have observed that in the liver of mammals, there is a positive correlation between the levels of mercury and its antagonist selenium (Koeman et al., 1973; Wagemann et al., 1996). Selenium can promote detoxification of methyl mercury (Hansen and Danscher, 1995). Mercury is stored in the liver in an insoluble, biologically inert form of inorganic crystals of Hg-Se (Caurant et al., 1996). However the corrosive acids of the human stomach may liberate Hg from the selenium complex and subject consumers to mercury exposure. Furthermore, selenium is present in servings of marine mammals dishes at levels which exceed the maximum tolerable daily intake (400 µg/day) (Yang et al., 1989).

According to Health and Welfare Canada (1984) mercury guidelines. Arctic coastal human communities exhibit levels above normal range. A 1988 study found that Inuit adults from one northern Québec village had mercury blood levels of 14.1 µg/l: In comparison, 80% of 50 Québec City adults tested had levels below 2 µg/l (Laliberté et al., 1992). An ongoing Canadian Arctic study has shown mean mercury concentration in cord blood samples from newborns to be 20 µg/l ranging from 0.6 to 214 µg/l (Ayotte et al., 1995). However, no biological injury appears to result from these high levels. The high level of selenium in marine food consumed by these populations may provide protection against methylmercury toxicity (WHO, 1990). The World Health Organization assesses a 5% risk of neurological damage for adults with blood methylmercury levels of 200 µg/l. A similar risk for offspring is associated with peak mercury levels of 40–80 µg/l in maternal blood (WHO, 1990).

Nordic residents of the Faroe Islands in the North Atlantic consume pilot whale meat which is contaminated with methylmercury (Grandjean et al., 1997). Children who were prenatally exposed to methylmercury (determined by MeHg levels in cord blood and maternal hair) underwent extensive neurobehavioural examination at age 7. The results suggest that brain

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function may be affected by prenatal exposure below a limit of 10 µg/g (50 nmol/g) for mercury in maternal hair (Grandjean *et al.*, 1997). Extrapolating from their data, researchers predict that a doubling of prenatal mercury exposure could cause a two-month developmental delay for several cognitive functions. If permanent, such deficiencies could have significant societal impacts on the affected populations.

The biological impact of methyl mercury contamination on Arctic marine organisms is unknown. As with all bioaccumulative contaminants, predators are most at risk of contamination via biomagnification. Previous studies have shown that piscivorous birds are particularly susceptible to methyl mercury poisoning from contaminated fish. Scheuhammer (1995) found that the dietary concentrations of methyl mercury required to produce significant reproductive impairment in adult birds were 20% of that sufficient to produce overt neurological symptoms. Dietary concentrations as low as 1–2 µg/g in fish prey have been shown to impair reproduction in free-living loons.

Sledgedogs may be a model system for studying the effects of dietary mercury exposure on humans. Hansen and Danscher (1995) studied a group of sledgedogs which were fed marine mammal organs and meat which contained bioaccumulated methyl mercury. This food was also consumed by the dogs' human owners, the Inuit of Thule, Greenland. Post-mortem analysis revealed that the contamination level in sledgedogs was comparable to that found in a group of Alsatians fed fish contaminated with methyl mercury for 7 yr (Hansen *et al.*, 1989). The distribution of methyl mercury in dog organs was similar to that described in Arctic seabirds, seals, whales and polar bears (Dietz *et al.*, 1990) The mercury concentration in the brain one of the oldest dogs was 1069 µg/kg. Dogs may be good sentinel species for humans even though humans tend to live at least 5 times longer than dogs and may be expected to bioaccumlate contaminants to potentially greater levels. In fact, a monitoring programme in Greenland measured high mercury levels (mean concentration 59–4782 µg/kg) in samples from Inuit brain autopsies (Mulvad *et al.*, 1996).

The human central nervous system is reported to be affected at a threshold concentration of 1280 µg/kg (Hansen and Danscher, 1995). The fact that dogs and the Inuit showed no signs of intoxication or malfunction may indicate that the high selenium levels in Arctic food may balance mercury toxicity by inactivating mercury via formation of insoluble mercury-selenide crystals. Continuing studies in the Arctic are needed to monitor the mercury levels and potential health effects in both the environment and Northern human communities.

Persistent Organic Pollutants

Sources of persistent organic pollutants

Many POP have no natural sources, such as PCBs and DDTs, and therefore provide unambiguous evi-

dence for the global extent of anthropogenic contamination (Lockhart, 1995). Other POPs, such as PAH and dioxin/furans have both natural and anthropogenic sources. Since the 1940s, POPs have been produced in large quantities for use as pesticides and industrial chemicals (Hargrave *et al.* 1992). The five major POPs in the Arctic environment are PCBs, DDT, HCH, chlordane and related compounds (e.g. PCC), and toxaphene (a mix of chlorinated bornanes and camphenes) (Muir *et al.*, 1992). PCBs have been used in capacitors and as lubricants, while DDT, HCH, chlordane, and toxaphene, have been used as pesticides (Barrie *et al.*, 1992).

Detection and measurement of POP residues in air, seawater, and marine organisms are complicated by the fact that each major group may contain many congeners or be a mixture of compounds. PCBs, chlordane, and toxaphene are complex mixtures of many chemicals with similar structures but different properties and toxicities while DDT and HCH are simple mixtures (Tanabe and Tatsukawa, 1986; Tanabe, 1988). These organic compounds are classified as persistent contaminants because they have long environmental half-lives due to resistance to chemical degradation by oxidation, reduction, hydrolysis, photolysis, free radicals, and other environmental chemical reactions (Tanabe and Tatsukawa, 1986). Scientists have known for decades that POPs can bioaccumulate in higher trophic level predators to concentrations which greatly exceed ambient air and ocean concentrations (Holden and Marsden, 1967; Robinson *et al.*, 1967; Jensen *et al.*, 1969). The first evidence of persistanent organic contamination in Arctic species was the detection in the 1970s of DDT residues in Arctic ringed seal (Holden, 1970). Although most of the POPs are agricultural and industrial chemicals that have been banned in North America and Western Europe for the past two decades, emissions continue today because of use in developing countries, improper storage and disposal (Ayotte *et al.*, 1995). Refer to Barrie *et al.* (1992), CACAR (Government of Canada, 1997), and AMAP (1998) for distal sources and inputs of POP that contaminate the Arctic. The AMAP Report also includes extensive tables detailing POP contaminant levels in soil, sediment, particulate matter, snow, sea ice, freshwater, saltwater, plants, terrestrial animals, birds, and marine organisms from numerous Arctic sites (1998). The remainder of this paper examines the transfer of POPs into the Arctic marine food web, the potential for biomagnification, and the consequences for the health of Northern human populations.

Transfer of persistent organic pollution to the Arctic ecosystem

As with acidifying gases and heavy metals, persistent organic contaminants enter the Arctic environment via global distillation: the volatilization of pesticides from temperate and tropical zones, followed by atmospheric transport northward, and redeposition of organic pollutants in colder regions (Goldberg, 1975; Patton *et al.*,

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1989) Many POPs are semi-volatile: substantial fractions of their mass may exist in the vapour phase, on atmospheric particles, adsorbed on the Earth's surface or dissolved in the sea (Barrie *et al.*, 1992). The partitioning depends on a compound's chemical properties, availability of particles for adsorption, and environmental conditions such as atmospheric temperature (Barrie *et al.*, 1992). The previously mentioned global fractionation model predicts that the most volatile POPs may be preferentially transported to the Arctic over less volatile POPs (Bidleman *et al.*, 1989; Wania and MacKay, 1993). This trend is demonstrated by the distribution of highly volatile HCHs. In a study of 22 tree bark samples from 90 sites worldwide, the environmental concentration of HCH was found to depend on latitude. Higher HCH concentrations were measured in tree bark from Arctic regions compared to equatorial regions (Simonich and Hites, 1995). Further evidence for this trend was provided by analysis of PCBs in an ice core from the Agassiz Ice Cap on Ellesmere Island, Canada (Gregor *et al.*, 1997). Due to limited melting in the summer, the annual ice layers undergo few chemical changes and thus provide a good record of historical contaminant deposition. Congeners with eight or greater chlorines are infrequently measured while the more volatile, lower chlorinated PCB congeners dominate the ΣPCB.

The global fractionation model also predicts that the preferential deposition of persistent, semi-volatile organics to the Arctic will be delayed and prolonged compared to temperate zones. Results of a study of PCB concentrations in lake sediments is consistent with this model (Muir *et al.*, 1996c). A 6-fold decline in ΣPCB flux over a latitudinal range of 35°N was observed. A higher proportion of lower chlorinated, more volatile PCB congeners were found in the Arctic. Analysis of lake sediment cores revealed that elevated ΣPCB and dioxin/furan (PCDD/PCDF) deposition began in the 1920–1940s in the mid-latitude and sub-Arctic lakes (Vartiainen *et al.*, 1997) while onset of contamination in the high Arctic was delayed until the 1950–1960s (Muir *et al.*, 1996c). Maximum ΣPCB concentrations were measured in sub-surface sediment dated to 1960–70s from southern lakes while high Arctic maxima is detected in surface sediment (Muir *et al.*, 1996c).

POPs have been detected in the Arctic air mass in the following order of abundance: HCHs (385–577 pg/m³) > toxaphene (44–36 pg/m³), PCB (14–20 pg/m³) > chlordanes (4–6 pg/m³) > DDT (< 1–2 pg/m³) (Bidleman *et al.*, 1989). The same trend of abundances of contaminants is seen in Arctic seawater. These patterns are related to each contaminant's Henry's law constant (HLC). HLC (a contaminant's equilibirum ratio of air to water concentration) determines if a contaminant exists mainly in the troposphere or in surface seawater (Murphy, 1984). For example, DDT has a low HLC and is rapidly transferred into water bodies near its mid-and low-latitude point source. Hence, DDT is relatively less

atmospherically dispersible over long distances which explains its low levels in the Arctic environment (Iwata *et al.*, 1993). In comparison, PCBs and chlordane have higher HLCs, tend to volatilize near tropical sources, and are transported long distances to the Arctic. The cold temperature of the polar region lowers the HLCs and favours deposition of PCBs and chlordane into Arctic surface waters (Iwata *et al.*, 1993).

HCHs are one order of magnitude more concentrated at the surface than at depth > 200 m (Bidleman *et al.*, 1989). PCCs were also found to have a distinct decline in concentration with depth; chlordane showed a less distinct gradient; and no trend was apparent for DDT and PCBs (Hargrave *et al.*, 1988). These higher contamination levels at the surface of the Arctic Ocean versus depth supports the hypothesis of atmospheric transport and deposition at the ocean surface versus transport via Atlantic Ocean deep water (Muir *et al.*, 1992). These trends are consistent with faster removal of more hydrophobic POPs via volatilization or sorption to sinking particles (Muir *et al.*, 1992). The residence time in the Arctic surface layer is estimated to be 10 years (Aagaard *et al.*, 1981; Ostlund and Hut, 1984) which suggests that organic pollutants may persist in the photic zone decades after their emission ceases.

Due to geographical and meteorological phenomenon, the Arctic is a global sink for volatile contaminants atmospherically transported from distal sources. But the Arctic may not be a permanent sink for organochlorines. The Arctic air-sea flux direction of HCHs has reversed from net deposition in the 1980s to net volatilization in the 1990s (Jantunen and Bidleman, 1996). This trend is due to a threefold decrease in atmospheric HCH levels as a result of global restrictions on usage (Jantunen and Bidleman, 1995).

Arctic sites of increased volatilization are the ecologically important melt holes and polynyas (large permanent open areas of water in pack ice). A study of PCB concentrations in the coastal plant saxifrage (*Saxifraga oppositofolia*) found levels to be significantly elevated at polynya versus non-polynya sites (France, 1997). Plants situated near low primary productivity polynyas have a higher proportion of higher chlorinated congeners compared to plants at ice-bound shores with high primary productivity. High productivity and thus sedimentation promotes the preferentially removal of higher chlorinated PCBs (Tanabe *et al.*, 1983). Higher contamination at polynyas is of concern because this microclimate provides a winter refuge for marine mammals and a spring staging ground for migratory birds (Sterling and Cleator, 1981; France and Sharp, 1992).

The Arctic Ocean has a low concentration of suspended particulate matter, especially below the upper mixed layer, which leads to low rates of sedimentation and increased residence time of particle-reactive organochlorines in the water column. The rate of sedimentation in the Arctic is comparable to that of the deep sea and enhances bioavailability of hydrophobic com-

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pounds compared to other oceans (Hargrave *et al.*, 1988). Particulate organic matter is bioavailable to fish, for example, while dissolved organic matter is not (see Gobas and Russel, 1991 for a simple model of bio-availability of organochlorines in water to fish). The POPs are readily adsorbed by living material (phytoplankton) or dead organic matter. When adsorbed to suspended particles, hydrophobic contaminants are available for consumption by grazing zooplankton and are rapidly removed from the water column and transferred to lipid-rich organelles and tissues (Hargrave *et al.*, 1988). Most persistent organic contaminants are sufficiently soluble in water to achieve ng/l concentrations which is adequately large to promote significant bioconcentration (Norstrom and Muir, 1994). The octanol/water partition coefficient (K_{ow}) provides an indication of a POP's affinity to become incorporated into the bottom of the food web. HCH has a low K_{ow} (10^4) compared to the more lipophilic chlordane and PCBs (10^5–10^8) (Mackay *et al.*, 1992; Iwata *et al.*, 1993). Although HCH is the dominant contaminant in the Arctic atmosphere and seawater, its low K_{ow} indicates that it would not be expected to be relatively concentrated in the food chain. In contrast, lipophilic PCC, chlordanes, PCBs, and DDT are expected to have significant bioaccumulative potential in Arctic organisms.

In addition to global atmospheric transport, POPs may enter the Arctic spawning lake ecosystem via migrating sockeye salmon (*Oncorhynchus nerka*). As muscle lipid deposits are depleted during their long migration, contaminants accumulated during the salmon's ocean life stage are concentrated in the lipid rich roe. Contaminants in salmon are more readily available for bioaccumulation than atmospherically deposited chemicals which are subject to adsorption to particles. Biotransportion permits long distance movement of nonvolative and short-lived contaminants which are not atmospherically transported. Arctic graylings (*Thymallus arcticus*) which scavenge salmon roe and carcasses in Alaskan lakes have higher PCB and DDT levels than graylings in lakes which do not host anadromous fish (Ewald *et al.*, 1998). The relative environmental significance of a contaminant is dependent on its persistence, the ability of organisms to metabolize and excrete it, and its efficiency of transfer between trophic levels (Norstrom and Muir, 1994).

Adaptation of organisms to the Arctic environment

The Arctic ecosystem supports seasonally low primary productivity, low species diversity and abundance. Arctic food chains are relatively short and are associated with simple predator-prey relationships from base to top predator: plankton-amphipods-Arctic cod-marine mammals-polar bears. Arctic organisms typically mature late, have low reproductivity, and are longer-lived than related temperate species (Barrie *et al.*, 1992). Physical factors such as low temperature, low solar radiation, large seasonal range in length of daylight, and

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ice cover that inhibits energy penetration contribute to food limitation in the Arctic environment (Muir *et al.*, 1992). Arctic organisms are adapted to exploit the short seasonal periods of high solar energy and food availability. These organisms rapidly consume and store organic matter in anticipation of periods of fasting when food is severely limited due to cessation of photosynthesis during the dark months (Hargrave *et al.*, 1992). Arctic fauna typically have high lipid levels in excess of 20% dry weight to protect against the cold and store energy for long periods of starvation (Bidleman *et al.*, 1989).

Persistent organic contaminants are typically highly lipophilic and resistant to biodegradation which allows them to bioconcentrate in the fatty tissues of Arctic fauna (Ayotte *et al.*, 1995). These chemicals biomagnify through the food web and tend to accumulate at higher levels in long-lived predators at the top of Arctic food chain such as seals, whales, polar bears and birds (Barrie *et al.*, 1992). The Inuits' traditional stragegy to survive the extreme Arctic cold was to adopt a high fat diet rich in calories and essential nutrients (Doubleday, 1996). Blubber and organ meats consumption exposes the Inuit to contamination. Persistent organic contaminants at high levels have been implicated in reproductive impairment of aquatic organisms (refer to reviews: Peterson *et al.*, 1993; Barron *et al.*, 1995; Brouwer *et al.*, 1995; AMAP, 1998). The life history characteristics of Arctic organisms make them vulnerable to both accumulation of chemical residues and to the toxic effects associated with the contamination.

POP body burden in Arctic marine fauna

The following sections describe how Arctic organisms may acquire a body burden of POP contamination originating from distant sources, and emphasizes POP biomagnification up the food web. Contaminant levels in an organism depend on its diet, position in the food web, ability to metabolize and excrete contaminants, age, sex, migratory and reproductive history (Norstrom and Muir, 1994). The first cases of organochlorine contamination in Arctic marine organisms were reported in the 1970s with the discovery of DDT, PCB, and toxaphene residues in Arctic seals (Holden, 1970; Holden, 1975; Andersson *et al.*, 1988) and polar bears (Bowes and Jonkel, 1975). Since that time, a large body of evidence of Arctic organochlorine contamination from primary producers to top predators has been collected (see compilated data in review by Muir *et al.* (1992) or AMAP (1998).) Several national and international data bases and specimen banks have been established to monitor contamination levels (AMAP, 1998).

Hargrave *et al.* (1992) detected residues of the five major classes of POPs (HCH, PCB, toxaphene, DDT, and chlordane) in plankton, amphipods and the glacial eelpout (*Lycodes frigidus*), an abyssal fish. The concentration of total POPs in zooplankton was approximately equal to that adsorbed on particulate matter. However,

when zooplankton of various sizes (<62 μm-2 mm) are compared on a lipid weight basis, the smaller plankton have 2-5 times greater contamination presumably due to an increased surface area to volume ratio. The levels in pelagic and benthic amphipods were similar to that in the eelpout liver which were an order of magnitude greater than zooplankton. Large benthic lysianassid amphipods (*Tmetonyx cicada, Anonyx nugax* and *Eurythenes gryllus*) accumulated higher concentrations on a dry and lipid weight basis than smaller species (*Onisimus* spp. and *Andaniexis* spp). Finally, long-lived scavenging amphipods, which may eat carcasses of fish and marine mammals, had 2-60 times the organochlorine contamination of plankton. These results were corroborated by another study which found that PCB and toxaphene levels in benthic amphipods were the highest of any organochlorines detected in Arctic fauna for that study (Bidleman *et al.*, 1989). In comparison to plankton which remain near the surface, abyssal shelf amphipods were found to have lower concentrations of HCH, and one to two orders of magnitude higher concentrations of DDT, chlordane, and PCBs (Hargrave *et al.*, 1992). This gradient is due to the highly lipophilic and therefore less water soluble nature of the latter chemicals which are more rapidly transported to depth on sinking particles than more water soluble compounds such as HCH.

Other bottom-dwelling Arctic animals have also been found to uptake organochlorines from sediment and may provide a POP source to higher predators (Bright *et al.*, 1995). Filter-feeding mussels (*Mytilus edulis*) and clams (*Mya truncata*) have PCB body burdens only slightly higher than the surrounding sediment. However sea urchins (*Stronglocentrotus droebachiensis*) which ingest sediment have PCB concentrations three to tenfold higher than the bivalves. Furthermore, sea urchins have limited ability to metabolize PCBs and thus may help elucidate the source of contaminantion. The benthic four horned sculpin (*Myoxocephalus quadricornis*) has PCB body burden in excess of levels previously published for Arctic char and Arctic cod (Muir *et al.*, 1988). Juvenile sculpins are an important prey item of the Greenland cod (*Gadus ogac*) and sculpin consumption may be a major route of contaminant exposure for other marine predators.

Hargrave *et al.* (1992) concluded that the majority of contaminants were accumulated by abundant, smaller organisms due to their relatively higher biomass in the marine ecosystem. POPs were transferred to larger predators in marine food web via prey consumption. Biomagnification, the partitioning of compounds between aqueous phase and fatty tissues of organisms, results because organochlorines are slowly metabolized and excreted. This pattern of POP distribution in Arctic fauna suggests that larger and longer-lived species that store lipids to survive prolonged periods of low food availability (e.g. seals, whales, polar bears) or that feed higher in the food web (carrion-feeding amphipods) are likely to accumulate the greatest body burdens.

In another food web study, PCB, chlordane, DDT, and toxaphene were found to contaminate Arctic cod (*Boreogadus saida*) muscle, ringed seal blubber and liver, and polar bear (*Ursus maritimus*) fat (Muir *et al.*, 1988). In many Arctic areas, the Arctic cod is the most abundant marine fish and an important prey item for marine mammals. This small (<300 mm), short-lived (5-6 yr), pelagic fish inhabits under-ice surfaces and is sustained by a diet of copepods (calanoids) and amphipods (*Paranthemistos* spp). The highest contaminant residues in Arctic cod were HCH (0.010 mg/kg wet wt.) and toxaphene (0.018 mg/kg) (Muir *et al.*, 1988).

Adult ringed seals feed predominantly on cod during ice covered seasons. During late spring and early summer, polar bears feed on the abundant supply of moulting ringed seals and pups. Bears are food-limited for the remainder of the year and undergo one of the most extreme fasts known to mammals (Polischuk *et al.*, 1995). During these periods of starvation, their adipose tissue is reduced from 40% to 50% to 10% of their body mass (Cattet, 1990; Pond and Ramsay, 1992) and organochlorines may be mobilized and affect target organs (Norstrom and Muir, 1994). A study of female polar bears during different stages of reproduction found that as adipose reserves decreased, organochlorine concentrations increased on a lipid weight basis in both adipose tissue and mothers milk (Polischuk *et al.*, 1995). Mother-to-cub contaminant transfers thus increase as the fast progresses. Exposure of cubs to contaminants during the critical early developmental stages could adversely affect their growth and survival.

As the top predator of the maritime food chain, polar bears are at high risk of persistent organic contamination from prey consumption. Furthermore, polar bears maximize their caloric intake by preferentially consuming highly contaminated skin and blubber of marine mammals (Letcher *et al.*, 1998). PCB residues on the order of 2-8 mg/kg in adipose and muscle and DDT levels an order of magnitude lower were observed by Bowes and Jonkel in a 1968-72 survey (1975). Soon after, Lentfer (1976) detected HCH and chlordanes residues in polar bears. A 1987-1994 survey of 900 marine mammal individuals found polar bears to be the most contaminated of all species (mean ΣPCB 25 ppm in fat) (Skaare *et al.*, 1994; Skaare, 1996). Polar bears POP profile may differ from other mammals because they can metabolize normally recalcitrant chlorinated aromatic compounds, including DDT metabolites and certain PCB congeners (Norstrom *et al.*, 1988). Dramatic bio-accumulation of PCB congeners occurred through the cod-seal-bear trophic link with levels from 0.0037, 0.68 and 4.50 mg/kg, respectively. Certain PCB metabolites (methylsulfone-PCBs) detected in ringed seal and polar bear were previously assumed to be solely formed by biotransformation of PCBs. New evidence suggests that polar bears may actually bioaccumulate many of their methylsulfone-PCBs body burden from consuming seals as only 7 of 24 methylsulfone-PCBs congeners demon-

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strate clear indications of being formed in bear (Letcher et al., 1998).

A recent study revealed that polar bears sampled from Arctic regions covered in permanent sea ice have higher ΣPCB concentrations than bears sampled from other regions (Norstrom et al., 1998). This geographical difference may be due to the under-ice-based feeding ecology of polar bear prey, the ringed seal. Bioavailable contaminants may be more important within the top few meters of water and within ice and snow melt, than in deeper waters (Norstrom et al., 1998). In areas of seasonal ice cover, the lowest trophic level is dominated by relatively uncontaminated phytoplankton and pelagic-feeding copepods. In contrast, more highly contaminated ice algae and under-ice amphipods are more important in regions of permanent sea ice cover. Similarly, a previous study attributed higher concentrations of cadmium in ringed seal and polar bear from the western Arctic permanent ice cover to a greater proportion of hyperiid amphipods in the ringed seal diet compared to the eastern Arctic with seasonal ice (Braune et al., 1991).

Persistent organic contaminants have also been detected in a variety of other marine mammals including northern fur seal (Callorhinu ursinus), bearded seal (Erignathus barbatus), harbour seal (Phoca vitulina), ringed seal, hooded seal (Cystophora cristata), walrus (Obdobenus rosmarus divergens), beluga, harbour porpoise (Phocoëna phocoena), and narwhal (Norstrom and Muir, 1994; Kleivane et al., 1995; Loewen et al., 1998; Nakata et al., 1998). Due to differences in feeding habits and migratory patterns, blubber contaminant levels were lowest in bearded and ringed seals < harbour seals < northern fur seals (Krahn et al., 1997).

Marine mammals are able to metabolize selected POPs by hydroxylation and dechlorination, but the highly chlorinated compounds (> 6 Cl) remain unmetabolised and bioaccumulate (Wolkers et al., 1998). Hence only a limited number of isomers are found in liver and fat of marine organisms (Vetter and Luckas, 1992). In mammals and birds, some chlordane, DDT and PCBs are metabolized to non-degradable compounds which are more persistent and toxic than their precursors (Norstrom and Muir, 1994). Of the five major POP classes, HCH is detected at a level an order of magnitude lower in marine mammals than levels for DDT, PCB, chlordane and toxaphene (Norstrom and Muir, 1994). On a fat weight basis, DDT and PCB contamination in walrus was detected on the order of <0.1–1.0 mg/kg. Contamination levels for cetacea were an order of magnitude greater (1–10 mg/kg) probably due to both less efficient metabolism and excretion of contaminants leading to biomagnification (Norstrom and Muir, 1994). A recent Norwegian study found alarmingly high PCB levels in harbour porpoises (mean ΣPCB 20 ppm in blubber) (Kleivane et al., 1995)

The total organochlorine body burden of marine mammals is clearly correlated to trophic level (Mössner

and Ballschmiter, 1997). Filter-feeding bowhead whales (Balaena mysticetus) are closer to the beginning of the food web and have low POP residues (Mössner and Ballschmiter, 1997). In contrast, predatory toothed whales and seals are at the end of longer food webs and have higher POP body burdens (Mössner and Ballschmiter, 1997).

Piscivorous birds are another group of top predators in the Arctic. Arctic-breeding birds that winter in Latin America, such as peregrine falcons (Falco peregrinus tundruius), have been known to be contaminated with organochlorine pesticides for sometime. PCB, DDT, mirex and dieldrin levels are higher in mature Arctic-breeding thick-billed murres (Uria lomvia) than in first year birds which have yet to travel to contaminanted southern overwintering grounds (Donaldson et al., 1997). Unexpected contamination was found in Arctic birds which do not migrate out of Canada to POP source areas, such as long-tailed ducks (Clangula hyemalis) (ΣPCB: 2.88–18.88, DDE: 0.61–3.75; dieldrin: 0.02–4.02) and black guillemots (Cepphus grylle) (ΣPCB:0.13–0.65, DDE: 0.10–0.24 ; dieldrin: 0–0.02) (mg/kg wet wt). (Johnstone et al., 1996). These species are ocean dwelling and feed on invertebrates and fish; contaminant exposure is hypothesized to be via the Arctic marine food chain. Contamination of these waterfowl species is elevated enough to cause its predator the peregrine falcon to have higher contaminant levels if it fed on waterfowl versus non-piscivourous birds or uncontaminated terrestrial mammals such as lemmings.

Biomagnification

The ratio of bioaccumulation to metabolism or biodegradation determines if a contaminant is stored in, for example, adipose tissue or eliminated from the animal. Biomagnification factors (BMF) are the ratio of a contaminant's concentration at one trophic level to that at the next lowest level calculated on a lipid weight basis. Muir et al. found that highly chlorinated PCBs had the greatest BMFs (1988). The average BMF between fish and seals for the DDT metabolite DDE was 62 for male seals and 18 for females. DDT and PCB contamination in males was also higher than females. The sex discrepancy is explained by the fact that females can depurate via reproduction and lactation. For chlordane, BMF from fish to seals was 6 and from seals to bears was 8. The finding that total DDT showed BMF of <1 for seals to polar bears has been interpreted as an indication that bears have a high capacity to metabolize DDT.

BMFs from water to higher trophic predators such as seals, cetaceans, polar bears or birds are on the order of 10^9 for PCBs. BMFs for HCH and PCC are two orders of magnitude lower, consistent with Arctic fauna's more rapid metabolism and excretion of these compounds compared to highly persistent PCBs (Muir et al., 1992).

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Trends in Arctic persistent organic pollution

The role of age and sex in POP body burden. Within one species, different contaminant levels are observed depending on the sex and age of the individual organism. Male marine mammals tend to be more contaminated by highly lipophilic compounds than their female counterparts (Skaare *et al.,* 1990; Skaare *et al.,* 1994; Kleivane *et al.,* 1995; Vetter *et al.,* 1995; Krahn *et al.,* 1997). In one study, male Arctic ringed seals were found to have DDT, DDE, and PCB levels in the low ppm range, twice as great as levels in females. This sex difference in contamination is likely due to excretion by females via lactation and parturition (Addison and Smith, 1974). Older females of the same age who differ in reproductive history may exhibit different POP body burdens (Muir *et al.,* 1996b). PCB and DDE residues were detected in Arctic pinniped milk (Bacon *et al.,* 1992). Lactation may be an excretion pathway for mature females as well as an exposure source for newborn seals. An initial decrease in POP residues from birth to sexual maturity followed by an increase during adult years is observed in marine mammals (Norstrom and Muir, 1994).

ΣPCBs and ΣDDT have been observed to accumulate with increased age in male polar bears, male harbour porpoises, and many species of male cetacea (Subramaninan *et al.,* 1988; Skaare, *et al.,* 1990; Borrell *et al.,* 1995, Kleivane *et al.,* 1995) but not in all populations of male belugas (Martineau *et al.,* 1987; Stern *et al.,* 1994; Muir *et al.,* 1996b). The lack of correlation of body burden with age in male belugas may be due to shifts in diets by older males (Muir *et al.,* 1996a). Age and diet may influence pattern of PCB congeners observed in seal blubber. Older male and female seals have significantly higher levels of highly chlorinated PCB congeners than younger seals due to dietary exposure. Immature seals (<2 yr) are sucked with their mother's milk which is contaminated with the more mobile, i.e. less chlorinated PCBs. Soon they begin feeding on amphipods which also have a higher proportion of the less chlorinated PCB congeners. Not until later do they consume Arctic cod which which is dominated by more chlorinated PCB congeners (Muir *et al.,* 1988). Arctic cod accumulate organochlorines via water-borne and dietary routes and may preferentially biotransform lower chlorinated PCB congeners via the cytochrome P450 detoxification enzyme system thus resulting in bioaccumulation of a larger proportion of highly chlorinated congeners (Muir *et al.,* 1988).

Congerner patterns based on age and sex are seen in polar bears. As they age, levels of lighter chlorinated PCBs in adult male polar bears decrease while slowly metabolized highly chlorinated residues increase. In contrast, both lesser and greater chlorinated contaminants decrease in concentration with age in female adult polar bears due to depuration via lactation and reproduction (Duffe and Norstrom, 1997). ΣPCBs were 46% higher in adult male bears than females persumably due

to PCB clearance via milk (Norstrom *et al.,* 1998). Polar bear cubs have twice the POP body burden of their mothers due to large seasonal dynamics in lipids in mature females. Female polar bears undergo four months of fasting during the end of their pregnancy and early lactation period. During this starvation period, fat is catabolized and POPs are mobilized and redisposed to target organs and cubs. Male polar bears also have increased levels of ΣPCB during the ice-free summer/fall fasting period compared to winter feeding times (Duffe and Norstrom, 1997). In this way, polar bears may be more at risk than initially indicated by levels of contaminants in bears sampled at times of relatively good nutritional status (Norstrom and Muir, 1994).

Temporal trends in Arctic persistent organic contamination. As there are few local sources of PCBs and DDT in the Arctic, body burdens of these organochlorines indicate the global scale of transport and distribution. Use of PCBs and DDT ceased in developed countries during the early 1970s. Monitoring Arctic organochlorine levels will reveal the time scale over which remote ecosystems can recover from exposure to these chemicals. (Muir *et al.,* 1997). Temporal trend data from Arctic species are limited. Only two programs of standardized, well defined, annually collected specimens have been undertaken for over 15 yr: 29 yr monitoring of fish and reindeer in the Swedish Arctic and sub-Arctic and 18–20 yr sampling of seabird eggs in the Canadian Arctic (AMAP, 1998). Temporal trend data of organochlorine burdens in other Arctic species are often difficult to interpret because they are often based on a small number of specimens collected during few (2–3) sampling occasions, sampling techniques were not standardized, and analytical methodology had changed, making comparisons with older data problematic (AMAP, 1998). Furthermore, individual and between-year variation are large (AMAP, 1998). To address this deficiency, the CACAR and AMAP reports recommend establishment of well-designed temporal trend studies which analyze abiotic and biotic samples from specimen banks created by the Canadian Northern Contaminants Program and its counterparts in the seven other Arctic nations (AMAP, 1998; Muir *et al.,* 1997). Summarized below are several decade-long studies which monitored organochlorine residues in Arctic lake fish, sea birds, ringed seal, and beluga.

Scandinavian databases are older than North American temporal studies. A 25 year long monitoring study of PCB residues in northern pike in sub-Arctic lakes of Finland found that PCB loading decreased from a maxima of 5–15 μg/g (lipid weight (l.w)) in the early 1970s to today's range of 0.5–3 μg/g (l.w). (Korhonen *et al.,* 1997). Since the late 1960s, temporal trend monitoring of DDT, PCB, HCHs and HCBs in biota has been conducted in Sweden. Significant declines of organochlorine body burden over time have been noted in herring, cod, pike, arctic char and guillemot egg (Bignert *et al.,* 1998).

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Since the mid-1970s, organochlorine levels have been measured in eggs and livers of adult Arctic sea birds. Organochlorine residues in eggs of black-legged kitti-wakes and thickbilled murres from Prince Leopold Is. have declined from the mid-1970s to late 1980s and then levelled off by the 1990s (Noble and Burns, 1990; Muir *et al.*, 1997). A similar pattern of temporal OC residue decline was detected in the livers of adult northern ful-mars (Nettleship and Peakall, 1987; Noble and Elliott, 1986; Muir *et al.*, 1997). These patterns of OC declines in these migratory bird species most likely reflect de-creased OC levels in North Atlantic overwintering grounds (Muir *et al.*, 1997).

Organochlorine body burdens in Canadian Arctic seals and whales have not declined as rapidly as residue levels in seabirds from eastern Canada (Addison and Smith 1974,1996; Elliot *et al.*, 1988). Long-term studies of organochlorine contamination in female ringed seal blubber have been conducted on Holman Island, Northwest Territories, Canada. ΣPCBs decreased by 40% between 1972 and 1981 and then leveled off at most recent sampling in the mid-1990s (Addison and Smith, 1974; Addison *et al.*, 1986; Addison, 1995). DDT-group pesticides in seal blubber decreased slightly over the same period with the greatest decline occurring during the 1980s rather than 1970s (Addison and Smith, 1974; Addison *et al.*, 1986; Addison, 1995). The lag in DDT decrease is presumably due to continued atmospheric input of DDT from Eurasian countries after the ban in western countries was imposed (Muir *et al.*, 1997).

Beluga blubber sampled from the Mackenzie delta region, Canada showed no significant decline in ΣPCBs, toxaphene, or chlordane over a 10 yr period nor decline in ΣDDT over a 20 yr period (Addison and Brodie, 1973; Muir *et al.*, 1997). Bioenergetics based bioaccu-mulation models predict that approximately 20 years or two generations time are needed to see changes in PCB body burden in belugas, due to the importance of in-tergenerational transfer and the slow elimination rate of contaminants (Hickie, 1995). Other contaminants with longer half-lives in beluga blubber will require more time for body burdens to decline (Muir *et al.*, 1996b).

In conclusion, DDT and PCBs have generally de-clined in examined Arctic biota from maximal levels detected in the 1970s (AMAP, 1998). However, tempo-ral data on other organochlorines, and representative sampling from many different regions of the Arctic, and data on body burdens in Arctic species not described above are lacking or insufficient to draw conclusions. These observations reinforce the importance of inter-nationally co-ordinated standardized sampling and archiving programs to monitor organochlorine con-tamination in the Arctic.

Arctic POP levels compared to other marine ecosys-tems. The limited geographic range of many Arctic species confounds scientists attempts at intraspecial comparison of contaminant levels worldwide (Vetter *et al.*, 1996). As an alternative, scientists compare body burdens between related species which reside in different geographic locations. The interspecial variation prob-lems with this approach are obvious.

Arctic contamination is greater than that in the Ant-arctic but much lower than all other ocean ecosystems. Antarctic krill have two orders of magnitude less chlordane body burden than Arctic amphipods (Har-grave *et al.*, 1992). While bulk plankton in the Arabian Sea has 10–1000×greater POP residues than Arctic plankton (Kannan and Sen Gupta, 1987).

PCB, chlordane, toxaphene, and DDT levels in Arctic cod muscle are elevated compared to Antarctic fish but are 2–5 times lower than fish from mid- and low-lati-tudes (Muir *et al.*, 1988). HCH levels in Arctic cod, however, are as high or higher than southern fish, re-flecting the relatively higher concentration of these compounds in Arctic waters (Muir *et al.*, 1988). Baltic ringed seals and Gulf of St. Lawrence beluga are 10–50 times more contaminated with DDT, PCBs, mercury and other POPs than their Arctic counterparts (Muir *et al.*, 1990; Wagemann *et al.*, 1990; Norstrom and Muir, 1994).

Toxaphene residues in Arctic seal blubber are an or-der of magnitude lower than those identified in seal blubber from the Baltic and North Sea blubber is one order of magnitude higher than Arctic samples (Vetter and Luckas, 1992). PCB levels in North Sea and Baltic seals are 30 times higher than residues in Arctic seals (Luckas *et al.*, 1990). In the 1970s, PCB, DDT and DDE levels in Arctic seals are one order of magnitude lower than other seals from eastern Canada (Addison and Smith, 1974). This difference has declined over the past two decades. In the 1990s, PCBs and total organochlo-rine body burdens in postpartum female seals from eastern Canada were twice the level of Arctic seals while DDT residues were similar among both populations (Beck *et al.*, 1994). Organochlorine contamination is 15 times lower in the Bering Sea/Arctic and eastern North Pacific species of marine mammals (northern fur seals, beluga, baleen bowhead whale) than western North Atlantic species (harbor seals, commmon dolphin (*Del-phinus delphis*), and pilot whale (*Globicephala melaena*)) (Mössner and Ballschmiter, 1997). Arctic beluga from Hudson Bay have a 30 times lower ΣPCB and ΣDDT body burden than the St. Lawrence River, Canada belugas which are exposed to local industrial sources of contaminants (Muir *et al.*, 1996a).

The concentration of chlordane, DDT, and PCBs in Arctic biota are lower than levels detected in sub-Arctic and mid-latitude marine organisms which are often ex-posed to local sources of contaminantion. For example, the concentration of ΣPCB and ΣDDT (270 000 and 161 000 ng/g, respectively) in blubber of ringed seal from the highly polluted Gulf of Finland was three orders of magnitude greater than residues in Bering Sea ringed seals (Krahn *et al.*, 1997). POP concentrations in Bering Sea ringed seals were 5 times lower than body burden measurements taken in Norway (Daelemans *et al.*, 1993)

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and 3–6 times lower than residue levels in Hudson Bay, Canada (Muir *et al.*, 1995).

The chlordane-related compounds are detected at levels comparable to DDT and PCB in fish and marine mammals only in the world's polar regions (Muir *et al.*, 1988). Elevated chlordane/ΣPCB and ΣDDT/ΣPCB ratios suggest an increased rate of input of chlordane and ΣDDT to the poles compared to other regions (Muir *et al.*, 1988; Vetter, *et al.*, 1995). Thus certain chemicals may be preferentially transported long distances to the pole and cause proportionally greater contamination than near source regions. The overall ranking of persistent organic contamination in seals and marine fish is highest in Atlantic Ocean, North Sea, Baltic > Northwest Pacific > Arctic Ocean > Antarctic Ocean (Muir *et al.*, 1988; Hellou *et al.*, 1993).

Detection of Currently Used Pesticides

In the 1970s, pesticides which contribute to persistant organic pollution began to be replaced in the Northern hemisphere by less stable organochlorine pesticides. The new class of pesticides were designed to be less environmentally persistent, to have lower environmental mobility, and to have minimal impact on the ecosystem. However, data from Chernyak *et al.* indicates that some members of this new class of pesticides have been transported to the Arctic (1996). Low levels of currently used pesticides were detected in the Bering and Chukchi Seas: chlorothalonil (220 pg/l) and trifluralin (1150 pg/l) in the surface microlayer; atrazine (400 pg/l) and chlorpyrifos (170 pg/l) in marine ice; and chlorpyrifos (19–67 pg/l) and endosulphan (5 pg/l) in seawater; and widespread detection of endosulfan in the polar atmosphere. The highest concentrations were detected in fog and sea ice. Fog can accumulate pesticides to unexpectedly high levels because its large surface area can scavenge gas and particle phase contaminants from the atmosphere (Seiber *et al.*, 1993; Hoff *et al.*, 1993). During the long periods of ice cover, the fog-derived pesticides accumulate on the ice and remain stable due to the cold temperature and low solar radiation. During the spring melt, the accumulated pesticides are released into the adjacent seawater over a short period. During this season, there is a high potential for ecological damage at the sea edge an ecologically important area. The concentration of currently used pesticides at some sites are elevated enough to be of concern. The toxicity of some of these pesticides on aquatic organisms approaches the highest values measured in the Chernyak *et al.* study: 0.07–0.17 µg/l 96 h LC$_{50}$ chlorpyrifos for *Gammarus lacustris* and 26–62 µg/l 96 h LC$_{50}$ trifluralin for rainbow trout (1996). Source reduction rather than new pesticide development may be the only true solution to the Arctic global contaminant problem.

The Effects of POP Body Burden on Arctic Animal Health

Levels of contaminants in organisms do not necessarily reflect their environmental levels if these chemicals are metabolized. Interspecial and interindividual differences in metabolic activity exists to complicate the issue of body burden. The ability of animals to metabolize chlordane or PCBs, for example, can be measured by comparing the residue levels of a persistant metabolite with levels of the parent compound. Norstrom and Muir (1994) determined the order of increasing capability to metabolize chlordanes and PCBs by Arctic consumers to be cod < narwhal = beluga < ringed seal < polar bear. Some chemicals are detoxified by metabolism while others are activated to more toxic forms. Thus the capacity of a particular species to metabolize chemicals may determine the risk associated with exposure to these chemicals.

Compared to southern species, relatively little is known about Arctic species' physiology and biological response to contaminants. The majority of studies describe POP residues in Arctic marine organisms but do not explore the possible effects of such body burden. This lack of analysis persists because little experimental toxicology has been conducted with Arctic species to determine dose-response relationships. Until such relationships have been established, scientists cannot assess the risk to Arctic organisms health associated with chemical exposure (Lockhart, 1992). Refer to De Guise *et al.* (1995) and AMAP (1998) for a review of the potential effects of marine contaminants on the reproductive system, endochrine system, immune function, and tumorogenesis in marine organisms.

Some scientists have proposed using biomarkers or the physiological response of an organism to a contaminant to help determine the ecological relevance of the pollutant. One such biomarker is the liver cytochrome P450 system in fish and mammals which can test for association between the level of POP inducers in the organisms and induction of P450 (Stegeman and Hahn, 1994; Bucheli and Fent, 1995; Lockhart, 1995). Cytochrome P450s are enzymes which metabolize lipophilic xenobiotics into more water soluble compounds which can be excreted. P450s may also activate contaminants to toxic intermediates and induction of P450s may disrupt the critical balance of steriod hormones or other endobiotics (Tanabe *et al.*, 1994). These enzymes are highly concentrated in the liver which is the most important organ involved in bioaccumulation and biotransformation of lipophilic compounds. The level and inducibility of P450s may be used as a measure of an animal's susceptibility to xenobiotic exposure.

One biomarker study found a striking correlation between cytochrome P450 induction in Arctic beluga liver and PCB blubber residues (Lockhart and Stewart, 1992). During the winter, these cetacea were trapped in a freshwater lake and lost approximately 200 kg body

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weight. During the fast, the whales mobilized body fats and concomitantly PCBs which may be responsible for the P450 induction observed. Although the correlation is not proof of causation, this study was the first statistical evidence that Arctic marine animals' current POP body burden may be associated with biological response (Lockhart, 1995). Corroborative evidence was provided by White et al. (1994) who noted that the Beaufort Sea beluga blubber concentration of mono-ortho and non-ortho PCBs was strongly correlated with the liver cytochrome P450 1A isozyme (CYP1A). More recently, results from Mössner and Ballschmiter (1997) suggest that higher POP residues in marine mammals leads to elevated levels of mono-oxygenases involved in PCB metabolism.

Another risk to Arctic animal health associated with contamination is adduction of important cellular components such as proteins, lipids, carbohydrates, or DNA. Organisms with high levels of DNA adducts may have altered genetic transcriptional function which may potentially result in a higher risk of toxicity, mutagenesis or carcinogenesis (Stowers and Anderson, 1985). A high proportion of polycyclic aromatic hydrocarbon (PAH)-DNA adducts were described in Arctic beluga whales which are removed from major sources of PAH contamination (Mathieu et al., 1997). PAHs, such as benzo(a)pyrene, are formed via combustion and are by-products of many industries. PAHs are atmospherically transported to the Arctic in the same manner as the previously discussed contaminants. Adduct were detected in 4 of 4 beluga kidneys tested, 9 of 12 brains, and 15 of 16 livers at levels ranging from 3 to 15.4, 7.6 to 140 and 3.1 to 164 adducts/10^8 nucleotides, respectively. If adducts occur in the germ line and cause mutations, there may be transgenerational consequences which might not be detectable for decades.

The polar bear is the top predator in the Arctic marine food web and may be an ideal sentinel species for monitoring levels and distribution of contaminants and may provide insight into the potential long-term consequences of pollution on human health (Bandiera et al., 1995). Norstrom et al. concluded that chlordane was the most acutely toxic POP detected in polar bears and that all other contaminants were at levels below which acute toxic effects could be expected (1988). However the polar bear is a long-lived animal and chronic exposure to low doses may have delayed effects. Induction of P450s may be used as a biological indicator for the functional exposure of polar bears to xenobiotics. Polar bears have been found to express high levels of the detoxification enzymes cytochrome P450s due to induction in response to xenobiotics (Bandiera et al., 1997). Cytochrome P450 1A induction was correlated with contamination by aryl hydrocarbon (Ah) receptor ligands such as TCDD, while cytochrome P450 2B was induced by ortho-chlorine-substituted PCBs and chlordanes (Bandiera et al., 1995; Letcher et al., 1996). PCB levels in Canadian and Norwegian polar bears have been reported in excess of 70 µg/g fat (Norstrom et al., 1988; Norheim et al., 1992) which approaches levels that are correlated to reproductive failure in other marine mammals (De Long et al., 1973; Addison, 1989). During seasonal fasts which may last several months, contaminants bioaccumulated in the fat may be mobilized and may concentrate in the liver. Mammals may be at greater risk from POPs during fasts (Bandiera et al., 1995).

Methylsulphone ($MeSO_2$), a metabolite of PCBs and DDE, has been detected in polar bears (Norstrom et al., 1988). Methylsulphone is a potent adrenocortico-toxin which interferes with normal corticosteroid hormone balance in mammals (Brandt et al., 1990). This contaminant has been suggested to be the cause of reproductive failure and jaw bone erosion in grey seal in the Baltic Sea in 1970s (Muir et al., 1988). Although reproductive failure in marine mammals has been linked to elevated organochlorine levels, no direct cause-effect relationship has been confirmed (Bacon et al., 1992). A recent study concluded that polar bears may not be at risk of direct immunotoxic effects from present environmental PCB exposure levels as measured by phagocytosis and lymphocyte proliferation. In vivo leukocyte functions were diminished by PCB concentrations greater than 20 ppm, a level which exceeds current polar bear contaminant body burden (Pagliarulo et al., 1997).

Several studies in non-Arctic seals have linked contaminant exposure to adverse health effects. One experimental study demonstrated poor reproductive success in harbor seals exposed to high levels of dietary PCBs (Reijnders, 1986). There is strong evidence that high organochlorine levels recorded in blubber of seals in the Dutch Wadden Sea have contributed to the low fertility among resident seals (Luckas et al., 1990). In one study of Baltic Sea ringed and grey seals, Bergman and Olsson (1985) concluded that chlorinated hydrocarbons were involved in a disease complex with symptoms ranging from reproductive failure to bone lesions to adrenal hyperplasia. This hyperadrenocortoicism was associated with high levels of PCBs and DDTs on the order of 1000 mg/kg and 100 mg/kg, respectively. Note that PCB and DDT levels in Arctic ringed seal blubber are >50 times below the apparent threshold for this disease (Bergman and Olsson, 1985).

In another study, Baltic Sea harbour seals were fed Baltic herring contaminated with potentially immunotoxic organochlorines (de Swart et al., 1996). Compared to seals fed uncontaminated herring, the experimental seals displayed impaired immune response including specific T cell responses and suppressed natural killer cell activity. Marine mammals with high levels of POP residues may be less immunologically prepared to cope with stressful stituations compared to animals with lower body burdens. Due to insufficient supporting biological, chemical, and epidemiological data, a cause-effect relationship between POP contamination and

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disorders in Arctic marine organisms cannot be made presently (Addison, 1989).

The Impact of Arctic Contamination on Human Health

The previously described bioaccumulation which leads to high levels of persistent organic contaminants in marine mammals may also be a risk to Arctic human communities. Northern inhabitants, especially Inuit and other aboriginal peoples who have traditionally lived and hunted marine mammals within the Arctic, derive a significant portion of their diet from so-called "country foods." To date 58% of 101 species of fish, wildlife and plants in the northern diet have been found to be contaminated with mercury, PCBs, toxaphene or chlordane (Chan, 1997). A traditional foods database has been developed to assess dietary contaminant exposure to chlordane, mercury, PCBs and toxaphene (Chan, 1998).

The dietary habits of Baffin Island Inuit women in the eastern Arctic and Sahtu Dene/Métis in the western Arctic were determined by quantified dietary recalls (Kuhnlein et al., 1995). Inuit rely on potentially contaminated marine mammals for sustenance with daily consumption by Inuit of different communities ranged from 10 to 160 g for ringed seal, 23–180 g bearded seal, 10–195 g/day muktuk, and 1–25 g walrus (Ayotte et al., 1995). In contrast, the Sahtu Dene/Métis communities consume relatively less contaminated caribou, fish and duck. Both population were found to be exposed to organochlorines through their diet. A small number of traditional foods of the Dene/Métis (arctic char, loche liver, trout flesh, and cisco flesh) contained sufficient concentration of contaminants to exceed tolerable daily intake levels (Berti et al., 1998). The Inuit women were more exposed to contaminants: 50% of dietary recalls exceeded the acceptable daily intake for toxaphene and chlordane-related compounds, and a large percentage of consumptive recalls exceeded the acceptable or tolerable daily intake levels for dieldrin and PCBs (Kuhnlein et al., 1995). On Broughton Island, NWT, 22% of men and 12% of women exceeded the medium-term tolerable daily intake of 1 µg/kg/day PCBs established by Health and Welfare Canada (Kinloch et al., 1992).

Ayotte et al. assessed the health risk of breast feeding on the body burden of dioxin-like compounds of Inuit from birth to 75 yr of age (1997). Simulations of a toxicokinetic model predict that breast feeding strongly influences body burden before but not after age 20. Although contaminant levels in fat and liver of Inuit are below threshold for health effects seen in laboratory animals, cancer and extreme reproductive defects, body burdens approach levels which may produce suble effects on the reproductive systems. Ayotte et al. concluded that a substantial portion of Inuit females would have fat concentrations of dioxin-like contaminants close to or higher than those associated with increased

incidence of endometriosis in rhesus monkeys (1997). However PCB congeners other than the non-ortho coplanars may anatagonize the effects of dioxin-like compounds.

Chan et al. developed a model to describe contaminant exposure in an Arctic human population from Qikiqtarjuaq on Baffin Island, Canada using Monte Carlo statistics to account for variations in diet contaminant concentrations due to seasonal hunting (1997). More than 50% of this indigenous population had dietary exposure levels that surpassed the World Health Organization's provisional or tolerable daily intake for chlordane, toxaphene and mercury (56%, 85%, and 73% for women and 71%, 91%, and 83% for men, respectively) (WHO, 1990; Chan et al., 1997). High end consumers, those in the 95% percentile, ingested 6 times the provisional tolerable weekly intake of mercury and over 20 times the tolerable daily intake of toxaphene and chlordane (Chan et al., 1997). The interactive effects of exposure to multiple contaminants is unknown.

Marine organisms contain high levels of omega-3 fatty acids. The omega-3 fatty acid content of plasma phospholipid in humans can be used as a surrogate measure for aquatic food consumption. Levels of omega-3 fatty acids, POPs, and heavy metals in blood sampled from 489 Inuit suggest that consumption of marine food may be an important source of exposure to organochlorines and mercury (Ayotte et al., 1995). Plasma samples from an Inuit population in Nunavik (Arctic Québec) had significantly higher mean total concentrations of PCBs (4.1 mg/kg lipids) and dioxin-like compounds (184.2 ng/kg lipids of 2,3,7,8-TCDD toxic equivalents) than plasma from a control population from Southern Québec (PCB: 0.13 mg/kg lipids; dioxin-like: 26.1 ng/kg lipids) (Ayotte et al., 1997). On Broughton Island, NWT, 29 of 46 children surveyed had blood levels of PCBs above the tolerable level of 4 µg/l kg established by Health and Welfare Canada (1992) (Kinloch et al., 1992). This level of contamination approaches that which induces adverse health effects in laboratory animals (Ayotte et al., 1997). The indigenous Arctic population's plasma contaminant load of PCBs and dioxin-like compounds increased with age and was greater in male than female subjects (Ayotte et al., 1997). Men have a greater contaminant body burden than women because adult males have a smaller adipose tissue compartment than females and females can depurate via lactation and reproduction. Due to their small stature, the Inuit reach contaminant levels of tolerance sooner than individuals with larger builds (Hild, 1995). Refer to AMAP for compiled data of contaminant levels in human hair, breast milk, urine, maternal and umbilical cord blood from the Nothern inhabitants of Arctic Council nations (1998).

As with polar bear cubs, cetacea and pinnipeds, children may be initially exposed to contaminants during gestation and upon consumption of their mother's milk (Dewailly et al., 1992). The mean total PCB load in

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breast milk of Inuit women was 3.6 μg/g milk fat, 5 times greater than southern Québec women with levels of 0.77 μg/g (Dewailly *et al.*, 1989). The PCB congeners found in breast milk reflect the geographic proximity to pollution source: short-lived PCB congeners predominated in southern breast milk while northern samples were enriched 10-fold with persistent PCB congeners (Dewailly *et al.*, 1993). A 1989–1990 survey found that the total 2,3,7,8 tetrachlorodizenzo-p-dioxin equivalents (TEqs: a measure of relative toxicity) for PCBs were 3.5 times higher in Inuit breast milk compared to southern woman (Dewailly *et al.*, 1994). The PCB levels in Inuit breast milk was the highest ever reported for a human population (Dewailly *et al.*, 1989). The levels of chlorinated pesticides or their metabolites (dieldrin, DDE, mirex, and hexachlorobenzene) in Inuit breast milk was 3–5 times higher than the southern samples (Dewailly *et al.*, 1993). Furthermore, the average cumulative duration of breast feeding among Inuit was 49 weeks compared with 12 weeks for their southern counterparts in this study.

Dewailly *et al.* (1993) compared the level of contaminants in breast milk from mothers in traditional Inuit settlement to more modern settlements. POP contamination in breast milk was 1.7 times greater for women from traditional villages which consume country foods compared to women from modern settlements where market foods predominate the diet. Furthermore, women from the traditional settlement breast fed for an average of 38 months compared to 14 months for the less traditional village. Not only were the children receiving a high dose of POP but for a period more than twice as long for their counterparts. High body burden of contaminants in Inuit mothers may bear public health consequences because of the ability of these compounds to cross the placental barrier and to bioconcentrate in milk fat, fetuses, and breast fed babies. To relate these levels to the Arctic marine environment, PCBs content in Inuit milk fat was similar to that of beluga whales and 7 times greater than that of Arctic char. Some highly persistent PCB congeners had even higher apparent BMFs between Arctic char and Inuit (BMF > 30). Thus Northern infants are one of the most heavily exposed group in the maritime food chain (Dewailly *et al.*, 1993).

Low doses of PCBs appear to adversely affect reproduction and neurodevelopment (Ayotte *et al.*, 1995) and promote an immunotoxic response to reduce total T-cell counts (Dewailly *et al.*, 1989). One immunological study of an Arctic community found that normal Inuit infants have lower total T-cell percentages and lower T-helper cells than a southern control group (Reece, 1987). Furthermore, rates of infection among Arctic children are 10–15 times those observed for southern controls. Dewailly *et al.*, suggest that Arctic contaminants may play a role in immunity impairment and subsequent infection rates (1989). The lower-chlorinated, less lipophilic congeners, which may be responsible for the neurodevelopmental effects, are not detected in the breast

milk of northern Québec Inuit women (Dewailly *et al.*, 1993).

These results warrant further studies to investigate the potential immunological and neurological effects of exposure to PCBs and mercury *in utero* and by diet in the Arctic. Any public health advice must consider both the potential risk associated with contaminant exposure versus the well known health benefits of breast feeding, the strenuous exercise associated a hunting lifestyle, and a nutritious country diet. In the 1970s, marine fat was discovered to have a preventative effect on atherosclerosis, thrombosis, and cardiovascular diseases (Dyerberg *et al.*, 1975). The low incidence of ischemic heart disease among Inuit was attributed to their high intake of fatty marine organisms. Traditional northern diets are rich in monounsaturated fatty acids, n–3 polyunsaturated fatty acids and selenium (as an integral part of the antioxidative glutathinoe-peroxidase enzymes) which may be the active agents which promote cardiovascular health (Dyerberg *et al.*, 1975; Bjerregaard, 1996). In addition, omega-3-fatty acids and selenium may respectively protect against PCB induced toxicity and mercury contamination (Ayotte *et al.*, 1995; Bjerregaard, 1996).

Presently no evidence links POP exposure in Arctic populations to increased cancer, liver disease, reproductive or developmental disorders (Kuhnlein *et al.*, 1995). However, some of the presistent organic pollutants may mimic hormones or growth factors and are found in Arctic populations at levels similar to that of physiological substances. Furthermore, mixtures of contaminants may have additive, synergistic or antagonistic effects which are as yet uncharactertized. Bjerregaard warns that in future we may discover transgenerational effects of POP contamination in the form of neurological, sexual, and developmental defects in the offspring of contaminated mothers (1995).

Receveur *et al.* (1997) compared the nutritional value of meals prepared by Dene/Métis communities in the Canadian Arctic using traditionally harvested foods versus market foods. Traditional meals were high in iron, zinc, and potassium and low in sodium, fat, saturated fat and sucrose compared to market foods. Market foods are often prohibitively expensive for northern residents and are of limited variety, freshness and nutritional value. Even if market foods were universally available they would not equal the nutritional benefit of a traditional diet. The transition from a subsistence to 'western' lifestyle and diet is associated with increased risks of cardiovascular disease, cancer, obesity, and diabetes in Baffin Island Inuit (Kinlock *et al.*, 1992). Changing diet to avoid contamination which poses potential but unknown hazards may put Northerners at risk for serious health problems associated with dietary deficiencies of protein, retinol, certain fatty acids, iron and zinc. CACAR and AMAP reports recommend that indigenous peoples continue to consume traditional food because their nutritional value outweigh the po-

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tential risk associated with chemical contamination (Government of Canada, 1997; AMAP, 1998). Additional research is needed to understand the long-term health effects of Arctic contaminants. For those who wish to lower their contaminant intake, CACAR recommends choosing traditional food lower in fat, e.g. fish and caribou, and to limit marine mammal fat intake by boiling and broiling meat.

Conclusion

Although the Arctic has no major point sources of anthropogenic pollutants, this remote environment is contaminated via long distance atmospheric transport of anthropogenic chemicals from mid- and low-latitudes. The level of contamination in Arctic air and water is many times less than contamination to the south, although it is greater than contamination in Antarctica. Many of these compounds are transferred from the atmosphere to the ocean milieu where they enter the bottom of the food chain via adsorbed by plankton. Plankton are in turn eaten by zooplankton, followed by fish, next seals and whales, then polar bear; amphipod scavengers may feed at all trophic levels. At each higher trophic level, the level of contamination increases. The top predator is humans who rely on marine mammals for much of their food, such as the Inuit, and are thus exposed to fairly high levels of contamination. The most highly exposed groups are human fetus and breast-fed infants whose mothers have eaten a Northern country diet of contaminated marine mammals.

Although, evidence of health effects in marine organisms and Northern inhabitants associated with contamination levels have not been conclusive, the contamination levels in and of themselves are worrisome. Remote areas are not isolated from anthropogenic pollutants by virtue of having no local point source: the ocean-atmospheric system can distribute contaminants throughout the biosphere. Due to consumption of local foods, circumpolar communities are analogous to miners' canaries in respect to global environmental health.

The Arctic is the ultimate global sink for many contaminants. Although the use of many contaminants has declined in some regions, the contaminant concentration in the Arctic environment continues to climb. For example, DDT and PCB levels in southern Canada peaked during the high use era of 1960–1970. This phenomenon occurs because residues continue to evaporate from soils in tropical regions and they are moved poleward via global distillation. Arctic contamination will persist for several decades after cessation of source emissions.

The only genuine solution is source reduction. But due to the global nature of the source of pollutants, efforts to limit contamination will be difficult. Developing countries in Eurasia which are currently the major emitters of persistent toxic insecticides, acidifying gases and heavy metals from their industrial plants may not

have the economic or political stability to limit their environmental impact. Furthermore, the Arctic itself is not one country which can act with a unified political voice. This vast scarcely populated remote area covers the northern reaches of three continents. A huge international will and effort is required to tackle this problem.

The resolution of the Arctic contamination problem requires multinational scientific collaborations to monitor global pollutants in the Arctic environment, marine life and human populations. In addition to chemical and body burden surveys, studies which determine the physiological effects of contamination are needed. The threshold limits of contaminants which induce chronic and acute effects on Arctic marine organisms and Northerners may then be established. The standardization of samples tissues, units, and dietary recommendations is needed to convey a clear, consistent message to policy makers and Northern communities. With this information, informed public health recommendations may be issued concerning whether the risk of contamination from a Northern country diet high in marine mammal content outweighs the benefits from selenium and omega-3 fatty acid content, exercise from hunting and the cultural importance of a traditional diet.

A major step toward these goals is the establishment in 1996 of the Arctic Council by the environment ministers of the eight Arctic nations: Canada, Denmark, Finland, Iceland, Norway, the Russian Federation, Sweden, and the US of America. The Arctic Council was established in part to oversee programs such as the previously established the Arctic Monitoring and Assessment Program (AMAP), conservation of Arctic Flora and Fauna (CAFF), and Protection of the Arctic Marine Environement (PAME). In 1997, AMAP released an Arctic pollution report, the culmination of 6 yr of studies by scientists from the Arctic nations, including the Canadian Arctic Contaminant Assessment Report (CACAR). Currently, controls on sources of POP and heavy metals in the northern hemisphere are being negotiated under the United Nations Convention on Long-range Transboundary Air Pollution. In 1998, negotiations for a global agreement on POP under the auspices of the United Nations Environment Programme will begin.

International meetings among northern First Nation peoples, such as the Inuit Circumpolar Conference established in 1977, have helped to promote awareness of the problem and provide a forum in which to discuss how to limit further Arctic contamination by southern sources. International governing bodies and institution such as the United Nations, World Health Organization, and World Bank, could use their influence to promote the use of low emission technology in all countries. In particular, World Bank loans and UN development money could incorporate environmental costs in economic models and favor funding industries

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in developing nations which are able to minimize their emissions.

The present contaminant levels in the Arctic are much lower than elsewhere in the world. However, even if contamination ceased today, many of the pollutants are resistant to biodegradation, will be transported to the Arctic via global distillation and will persist in the northern environment for decades to come. The Arctic experience demonstrates the global effect of human activity.

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16. Abstract (Limit: 200 words)

P-glycoproteins, responsible for multidrug resistance, function as energy dependent efflux flippases that prevent the cellular accumulation of moderately hydrophobic chemicals. We characterized P-gp expression in populations of several fish species exposed in their natural habitat to environmental contaminants which may be P-gp substrates/inducers. We evaluated whether P-gp activity may be implicated in this multixenobiotic resistant phenotype. P-glycoprotein levels were elevated in tissue surrounding liver tumors in winter flounder (*Pleuronectes americanus*). Killifish (*Fundulus heteroclitus*) from a contaminated field sites had higher intestinal P-gp and lower hepatic P-gp than control killifish. P-glycoprotein was induced in fish by oil exposure but not by ß-naphthoflavone or 2,3,7,8-tetrachlorodibenzofuran. Thus, P-gp expression is not regulated by the aryl hydrocarbon receptor pathway. We developed a protocol for an *in vivo* assay to simultaneously evaluate P-gp-mediated transport of a model substrate, rhodamine B (rhB), in multiple organs of killifish. Our results indicate that P-gps play a major role in transport of xenobiotics in fish expecially in liver, brain, and ovary. Using this assay, we assessed whether the common environmental contaminant and carcinogen benzo[a]pyrene (B[a]P) is a P-gp substrate. We show that B[a]P and/or its CYP1A metabolites are not transported by P-gp in liver, brain, or ovary.

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